The role of hormones and ions in the physiological control of cellular proliferation in rat erythropoietic and lymphopoietic tissues.

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by

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SUMMARY

Physiological situations are described in which changes in the mitotic activities of rat bone marrow and thymus are precisely regulated by modulations of the calcium homeostatic system. In this context, other hormones are demonstrated to interact with those controlling calcium homeostasis. Erythropoietin and the anti-diuretic hormone have been studied in depth and are shown to be co-ordinated with the parathyroid gland in the genesis and maintenance of the enhanced bone marrow mitosis which is necessary to restore lost red cell mass. Observations *in vitro* have led to hypotheses to explain the mechanisms of action of these hormones.

Circadian variations in bone marrow and thymus mitotic activity are shown to closely parallel shifts in plasma calcium concentration. These calcium changes are probably dependent upon fluctuations in the activity of the parathyroid gland.

The mitogenic properties of exogenous erythropoietin are largely mediated via elevations in calcium in the plasma. The bone marrow response to haemorrhage is dependent upon the co-ordinated actions of parathyroid hormone and calcium, erythropietin and anti-diuretic hormone. Interrelationships between other erythropoietic stimulants or inhibitors and calcium homeostasis have been briefly investigated in the whole animal.

Studies using *in vitro* bone marrow cultures suggest a role for cyclic adenosine 3', 5' - monophosphate in the intracellular mediation of mitogenic stimulants.

Proliferative activity in the thymus following erythropoietic stimuli has a number of parallels with that in bone marrow. The functional significance of this observation is not clear.

Thus the hormones of the calcium homeostatic system, via their ability to modulate body mobile calcium, constitute an important control factor in the physiological regulation of cell proliferation in bone marrow and thymus.

I hereby certify that no part of the work reported in this thesis was performed in collaboration, except for a section in Chapter 2 which is clearly acknowledged in the preface to that chapter. No part of this work has been submitted for the award of any other degree.

N.H. Hunt

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GENERAL INTRODUCTION

"Those experienced in work must take up the study of theory and must read seriously; only then will they be able to systematise and synthesize their experience and raise it to the level of theory, only then will they not mistake their partial experience for universal truth and not commit empiricist errors....."

Mao Tse-Tung.

General Introduction.

This study has been concerned with delineating a number of control factors and hormonal influences upon cell division in haemopoietic and lymphopoietic tissues in the (Wistar strain) albino rat. This introduction outlines the basic phenomena and problems of the maintenance of balance of blood cells and thymocytes, and describes the physiological systems which have been implicated as regulatory mechanisms. Finally, the observations which suggested the lines of investigation which were ultimately to be followed are discussed in depth.

The material which is presented in this and other sections of this study may be assumed to apertain to the rat unless qualified in the text. Experimental observations in other animals have been used as background information alone, since the complicating species distinctions between the various systems which have been considered make extrapolation, even among the Mammals, a suspect and often misleading process.

The consideration of the cell cycle has been deferred to the General Methodology (Sections 6.2 and 6.3) where it is elaborated in terms of the methods used for measurement of mitotic activity in test tissues. Specific aspects of the literature pertinent to limited areas of the study are discussed in the Introduction to individual chapters of the Results section.

1. Commonality of Origin of Blood Cells.

Mature, circulating blood cells are both highly differentiated and, except for some types of lymphocytes, incapable of division. Since their life span is usually short compared to that of the animal constant replenishment is demanded. In adult rats this is achieved by proliferation, differentiation and maturation of precursor cells in the bone marrow, thymus and peripheral lymphoid tissues. In foetal or newly post-partum animals there is additional involvement of the yolk-sac, liver or spleen, depending upon the stage of development (c.f. Cole & Tarbutt, 1973).

Although the different types of blood cells demonstrably differ both functionally and morphologically there is now little doubt that they are ultimately derived from a common precursor cell (Lajtha, Oliver & Gurney, 1962; Lajtha, 1963; Wu, Till, Siminovitch & McCulloch, 1967, 1968; Lajtha, 1970). The concept of this precursor, which has been termed the pluripotential stem cell, is discussed later (Section 2.10), following descriptions of basic observations in the areas of major interest, i.e. the erythroid and lymphoid systems.

2. The Nature of Haemopoiesis.

2.1 Red Cell Balance.

A striking observation common to many haematologists is the remarkable constancy of the circulating red cell mass in normal conditions. This phenomenon, coupled with the ability of the animal to adjust red cell numbers in response to pertinent environmental stimuli, suggests the presence of an efficient and sensitive control system. In steady conditions this control achieves a balance between the twin processes of red cell (erythrocyte) production and destruction, which implies that overall control may be exerted by a combination of at least two systems which are integrated to maintain the *status quo* or provide adaptation to changing conditions.

The ratio of erythrocyte life-span to production time is large, and a regulating system with the prime component directed upon the modification of red cell destruction rate would thus be insensitive to short term stimuli. For this reason the factors influencing the rate of production of erythrocytes have been considered to be of prime importance and have been extensively studied, particularly within the last two decades.

It is not possible to fully appreciate the nature of erythrocyte production without consideration of the functions of the mature red cell. These are therefore briefly discussed in the subsequent section.

2.2 Erythrocyte Function.

The major roles of the red cell are the transport of oxygen from lung capillaries to metabolising tissues and the carriage of carbon dioxide in the reverse direction. The cell is highly specialised for these functions. The biconcave, disc-like shape is considered to facilitate oxygen-haemoglobin interaction (Valtis, 1955) and there is

a high (up to 95% of total protein) haemoglobin content (Harris & Kellermeyer, 1970b). The manifold physiological advantages of "packaging" haemoglobin within a circulating cell structure have been reviewed by Harris & Kellermeyer (1970b). Summarised, these are:

(a) Haemoglobin dissolved in plasma has a half-life of only some 3 hours, compared to many days within the erythrocytic membrane. The life span of the rat erythrocyte has been estimated at 50 - 60 days (see Berlin, Waldmann & Weissmann, 1959 for review).

(b) The haemoglobin is in close proximity to a number of enzyme systems which serve to maintain it in the chemical form required for efficient oxygen transport.

(c) Sequestration of haemoglobin within the corpuscle permits oxygen carriage without the necessity of tolerating high osmotic pressures, which would occur if the same amount of haemoglobin was in solution in the plasma. Thus the osmotic pressure of plasma is only some 20% of that of a solution of the "packaged" haemoglobin in plasma.

(d) "Bolus" flow in capillaries is probably more efficient than laminar flow, since there are no stagnant areas adjacent to vascular walls.

The intricate processes which produce these highly specialised respiratory gas carriers from their precursor cells are termed erythropoiesis, and will be considered in a later section in the light of the control systems which have been postulated.

2.3 Destruction of Erythrocytes.

As mentioned in Section 2.1, control of the rate of destruction of erythrocytes is not considered to be of primary importance in the short-term control of red cell production in reponse to environmental changes. However, in the long-term maintenance of circulating red cell

mass it is clear that a distinct regulator of cell death must exist. Unfortunately, the determinants of erythrocyte life-span under normal circumstances are not fully understood. The factors which ultimately lead to the destruction of the cell in other species have been variously postulated to be increased susceptibility to osmotic (Hoffman, 1958; Danon, 1961) or immune (Griggs & Harris, 1961) haemolysis, or intracellular accumulation of noxious metabolites (Harris & Kellermeyer, 1970b), i.e. to changes related to the biochemical and biophysical characteristics of the individual erythrocyte.

It is known that natural erythrocyte destruction in the rat is accomplished chiefly within the red pulp of the spleen (Weiss, 1970b), though sequestration and destruction may take place in the liver, and intravascular death also occurs.

2.4 Investigations of the Possible Humoral Control of Erythrocyte Production.

In 1906, Carnot and Deflandre claimed that injection of small quantities of serum from anaemic rabbits into normal recipients produced a doubling of red cell count within 3 days. They postulated that this increase was due to a humoral factor, responsible for increasing red cell production, which was present in supranormal quantities in the plasma of the anaemic donors. This factor was termed "hémopoiétine". Subsequent studies, notably by Erslev (1953), showed that such responses to anaemic plasma injection were unique. Increased red cell count is in any event a poor erythropoietic criterion since haemoconcentration may elevate the numbers of circulating erythrocytes per unit volume without any occurrence of increased erythrocyte production. Thus, as observed by Borsook in discussion, the concept of a humoral regulation

of erythropoiesis was maintained for 40 years by faith rather than by unequivocal demonstration.

A series of investigations during the 1950s brought the concept of a humoral erythropoietic factor to the point of acceptance. In an elegant experiment Reissmann (1950) demonstrated that when one of a pair of parabiotic rats was subjected to a low oxygen tension, while the other was maintained under normal conditions, increased erythropoiesis resulted in both parabionts. Hyperplasia of the bone marrow was the erythropoietic criterion, since increases in circulating reticulocyte or erythrocyte numbers would obviously have been transferred between the parabionts. Shortly after this study, Stohlman and his colleagues (Stohlman, Rath & Rose, 1954) reported a case of polycythemia in man secondary to a patent ductus arteriosus with reversal of arterial flow. Thus a regional hypoxia existed below the diaphragm, with normal arterial oxygenation above. Erythroid hyperplasia existed in both areas, indicating that anoxia did not stimulate erythropoiesis by a direct action on bone marrow. Schmid and Gilbertsen (1955) later reported a similar case.

Direct demonstrations of a humoral erythropoietic factor were provided by several workers in the early 1950s (Borsook, Graybiel, Keighley & Windsor, 1954; Crafts & Meineke, 1956; Erslev, 1953; Gordon, Piliero, Kleinberg & Freedman, 1954; Hodgson & Toha, 1954; Plzak, Fried, Jacobson & Bethard, 1955; Prentice & Mirand, 1956). The general approach was to inject into normal rats serum from rabbits rendered anaemic by bleeding. Polycythemia, reticulocytosis or uptake of ^{59}Fe into peripheral red cells were used as measurements of erythropoietic activity. The erythropoietically active humoral fraction has been termed erythropoietin (EPO), erythropoiesis-stimulating factor (ESF) or haemopoietin. The latter term is misleading since the factor specifically acts to increase erythrocyte numbers and not, as far as is known, any of the complex constituents or other cell populations of "haem". The

term erythropoietin has been adopted in the present study since it is specific and brief.

2.5 Fundamental Stimulus for Erythropoiesis.

In most circumstances, the observed stimulus for increased erythropoiesis has been anoxia produced by one of several means (Grant & Root, 1952). This observation has been conceptualised by Jacobson and his co-workers (Fried, Plzak, Jacobson & Goldwasser, 1957), who contended that the ratio of oxygen demand to supply governed the production of EPO and therefore erythropoiesis. Thus, it is postulated that in anaemic states EPO production is stimulated by the reduction of the oxygen-carrying capacity of the blood while tissue demand remains constant, and, conversely, the increased oxygen-carrying capacity of polycythemic blood causes decreased EPO production. Furthermore, when tissue oxygen demands are increased (e.g. by exercise) or decreased (e.g. by starvation) in the presence of a normal circulating red cell mass EPO production is stimulated or depressed, respectively. Note that this concept implies the existence of an oxygen supply/demand sensor.

The above hypothesis has received a great deal of support from studies in a variety of mammalian species. In the rat:

(a) a direct relationship between the prevalent degree of anaemia and the plasma EPO titre has been established (Eskuche & Hodgson, 1962);

(b) polycythemia (Jacobson, Goldwasser, Plzak & Fried, 1957a), hyperoxia, and reductions in metabolic activity following hypophysectomy or starvation all reduced erythropoiesis and, indirect evidence suggests, circulating EPO levels (Jacobson & Goldwasser, 1958); and

(c) agents that increased the metabolic rate and therefore the tissue oxygen consumption, such as thyroid hormone (Meineke & Crafts, 1959) and dinitrophenol (Jacobson & Goldwasser, 1958), stimulated erythro-

poiesis and again, by inference, circulating EPO levels.

Despite these observations, it seems possible that the mechanism postulated by Jacobson does not fully explain the regulation of erythropoietin production. For example, a number of compensated haemolytic syndromes have been described (Stohlman, 1959) in which red blood cell destruction and formation are greatly increased but balanced. In these circumstances plasma levels of EPO are not demonstrably increased (Erslev, 1964). However, this could be ascribed to the insensitivity of EPO assay procedures (Krantz & Jacobson, 1970a) or increased sensitivity of bone marrow to the hormone (Erslev, 1964) rather than to a second regulatory system involving a feedback mechanism from peripheral red cells (Stohlman, 1962). It is safe to conclude that the great majority of erythropoietic responses to various stimuli are mediated via increased EPO activity, produced in response to changes in the ratio of tissue oxygen supply to demand.

As mentioned above, it is necessary to visualize a sensor of oxygen supply/demand ratio. The possible nature and location of this can best be considered after discussion of the sites of EPO production.

2.6 Sites of Production of Erythropoietin.

Initial investigations to locate the site(s) of production of EPO were conducted empirically by means of organ excisions and preparation of tissue homogenates or extracts. The exhaustive studies of Gordon and his colleagues (Gordon, Piliero, Medici, Siegal & Tannenbaum, 1956; Gordon, 1957) demonstrated that extracts of liver, spleen, lung, bone marrow, thymus, brain, muscle and erythrocytes, all derived from anaemic rabbits, had no detectable erythropoietic activity. In addition, removal of pancreas, thymus, adrenals, stomach, gonads, spleen or intestines were shown to have no effect upon the response of animals to the

erythropoietic stimulant cobalt (Jacobson, Goldwasser, Fried & Plzak, 1957b). The effect of hepatectomy was equivocal (Jacobson, et al., 1957b; Katz, Cooper, Gordon & Zanjani, 1968). The pituitary has been postulated to produce an erythropoietic factor (Contopoulos, Van Dyke, Simpson, Garcia, Huff, Williams & Evans, 1953), but this thesis has been shown to be untenable by subsequent investigations (Fried, Plzak, Jacobson & Goldwasser, 1956; Halvorsen, Roh & Fisher, 1968). The influence of the pituitary upon erythropoiesis is discussed later (Section 2.12).

The classic experiments of Jacobson and his associates (Jacobson et al., 1957b) demonstrated that bilateral nephrectomy in rats inhibited the erythropoietic response to various forms of hypoxia. Control animals were subjected to ureteral ligation and the subsequent uremia marginally reduced the erythropoietic response to hypoxia, though very much less than did nephrectomy. Subsequent investigations have unequivocally confirmed that an intact kidney is necessary for normal erythropoietic control in the rat (Jacobson, Goldwasser, Gurney, Fried & Plzak, 1959; Reissmann, Nomura, Gunn & Brosuis, 1960). This is also true in several other species, including man, mouse, rabbit and dog (Fisher, 1972).

Although the kidney is the major organ necessary for the formation of EPO, a number of studies have demonstrated the existence of extrarenal sites of EPO production (Mirand & Prentice, 1957; Mirand, Prentice & Slaunwhite, 1959; Halvorsen et al., 1968; Mirand & Murphy, 1970). This extrarenal EPO is similar to kidney EPO since it is neutralised by the antiserum to the latter (Fried, Kilbridge, Krantz, McDonald & Lange, 1969). Hepatectomy has been reported to abolish extrarenal EPO production in rats (Fried, 1971) and presumably therefore plays a major role in this production. However, it seems that the liver is a likely site for the synthesis of a plasma substrate which is acted upon by an enzyme to produce EPO (see below). Therefore the

observations of Fried cannot be taken as evidence that the liver is a site of extrarenal EPO production. Recently the carotid body has been suggested as another possible site of EPO production in cats (Tramazzani, Morita & Chiocchio, 1971). These authors have further suggested that his organ exerts overall control of erythropoietic activity. Some scepticism has been expressed towards their data (Erslev, 1971b)and, indeed, diametrically opposed results have been obtained in rabbits (Paulo, Fink, Roh & Fisher, 1972a), man (Lugliani, Whipp, Winter, Tanaka & Wasserman, 1971), rat (Beynon & Balfour, 1973) and even in cats (Paulo, Fink, Roh & Fisher, 1972b; Gillis & Mitchell, 1973).

Although the existence of extrarenal sites of EPO production in the rat is indisputable, their physiological significance is a matter of some conjecture (Gordon & Zanjani, 1970). It is possible that they are only operative in the adult as a compensatory mechanism after nephrectomy or gross renal damage. In this context, it is interesting to note that in the dog EPO production is completely kidney-dependent (Naets, 1960a; Mirand, Murphy, Bennett & Grace, 1968). However, EPO production in neonatal rats is probably largely from extrarenal sites (Carmena, Howard & Stohlman, 1968).

The observations of a number of workers (Fisher & Birdwell, 1961; Halvorsen et al., 1968; Kuratowska, Lewartowski & Lipinski, 1964; Pavolvic-Kentera, Hall, Bragassa & Lange, 1965), namely that perfusion of kidneys with hypoxic or cobalt-containing blood resulted in increased EPO titres in the perfusate, have clearly demonstrated that, in the rabbit and dog at least, the kidney is the major site of EPO production. These workers were unable to substantiate the assertion (Erslev, Solit, Comishion, Amsel, Ilda & Ballinger, 1965) that EPO is released *in vitro* only from injured or disintegrating renal tissue. The technical problems of renal perfusion in the rat have frustrated attempts to

extend the above observations to this species. Therefore it is possible to conclude only that, in the rat, the kidney is the major organ controlling EPO production. The available evidence does, however, strongly infer that this organ is also the site of formation of EPO.

Attempts to extract EPO from renal homogenates have met with only partial success in a number of species (Goldfarb & Tobian, 1963; Naets, 1960b; Rambach, Alt & Cooper, 1961; Zangheri, Suarez, Campana, Silva & Ponce, 1962). In addition, the low erythropoietic activity of the homogenates could have been attributable to the hormone content of trapped plasma. The definitive experiments of Kuratowska (Kuratowska et al., 1964; Kuratowska, 1965) demonstrated the existence of a renal factor capable of generating an erythropoietically-active substance when incubated with plasma or α-globulin. Thus she postulated (Kuratowska, 1968) that the kidney produced a factor (erythrogenin or renal erythropoietic factor — REF) which acted upon a plasma α-globulin substrate to generate EPO. Gordon and his colleagues were able to extract the REF from rat kidneys (Contrera & Gordon, 1966; Contrera, Gordon & Weintraub, 1966) and demonstrate that it was enzymatic in nature (Zanjani, Contrera, Gordon, Cooper, Wong & Katz, 1967a). They have since reported that the light mitochondrial fractions from rat kidney glomeruli, tubules, medulla and cortex contain the REF (Gordon, Cooper & Zanjani, 1967; Zanjani, Cooper, Gordon, Wong & Scribner, 1967b) and that an antiserum developed against the REF inhibits erythropoiesis in mice (McDonald, Zanjani, Lange & Gordon, 1971). The site of production of the plasma substrate (erythropoietinogen) in rats is probably the liver (Katz et al., 1968), but the intrarenal site of REF production is unclear. Several studies have indicated that the juxtaglomerular apparatus (JGA) is a possible site for the interaction of the REF and erythropoietinogen (Hirashima & Takaku, 1962; Takaku,

Figure 1 overleaf.

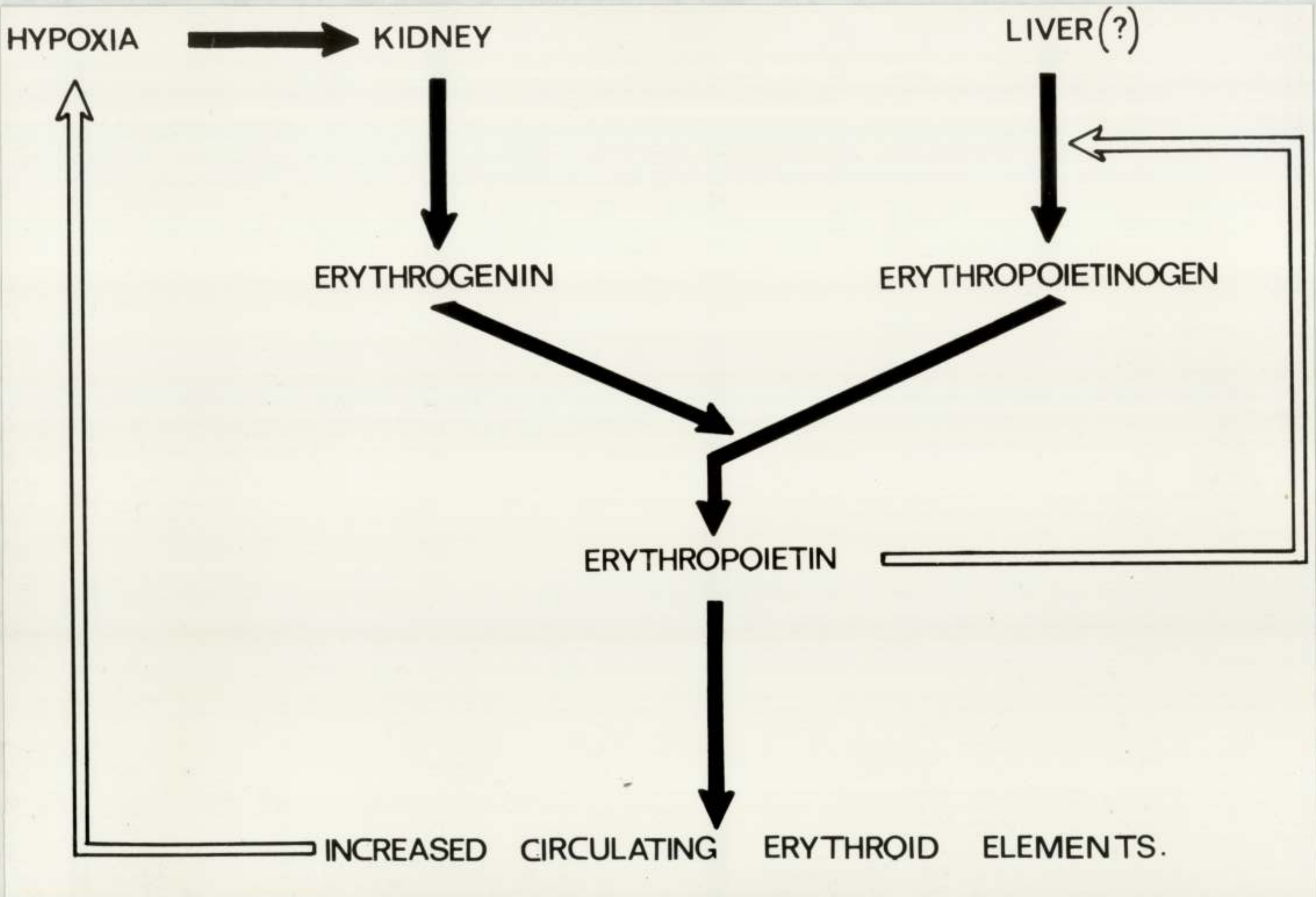


Figure 1: Biogenesis of EPO. Slightly modified from Gordon & Zanjani, 1970. Nomenclature discussed in text.

Hirashima & Nakao, 1962). However there are great difficulties in ascribing changes in JGA cell granularity to an erythropoietic response as distinct from a facet of the renin-angiotensin system (Gordon & Zanjani, 1970). In addition, it is possible that demonstrable EPO (e.g. by fluorescent-labelled antibody techniques) may represent stored or trapped, rather than locally formed, hormone.

2.7 A Scheme for the Control of Erythropoiesis.

A cogent hypothesis for the control of EPO production and erythropoiesis has been developed by Gordon & Zanjani (Gordon, Zanjani & McLaurin, 1971). This scheme is illustrated in Figure 1. Hypoxia is considered to be the primary stimulus which induces REF (erythrogenin) formation in kidney sites. The enzyme then acts upon the plasma substrate (erythropoietinogen), within the kidney or the circulation or both, to yield EPO which acts to increase formation of erythrocytes. A possible stimulatory role for hypoxia upon substrate formation is unproven (Gordon & Zanjani, 1970). However, these authors postulate a negative feedback action of increased circulating EPO levels upon substrate formation, in addition to the feedback constituted by the reduction of hypoxia by increased numbers of circulating oxygen-transporting red cells. This scheme obviously has some striking similarities to the renin-angiotensin system and it is tempting to postulate some connection between the two mechanisms. However, a number of studies in rats, mice and rabbits (Donati, Bourgoignie, Kuhn, Gallagher & Perry, 1968; Bilsell, Wood & Lange, 1964; Zanjani, Contrera, Cooper, Gordon & Wong, 1967c; Gould, Keighley & Lowy, 1968) have demonstrated that they are superficially analagous but functionally distinct systems.

The production of EPO by kidneys perfused with hypoxic blood (Fisher & Birdwell, 1961; Fisher & Langston, 1967; Halvorsen et al.,

1968; Pavlovic-Kentera et al., 1965) strongly implies that some sensor of oxygen supply/demand ratio exists in the kidney of dogs and rabbits. However, this might not be the main regulator of REF formation in the intact animal, as suggested by Fisher (1969), but merely a secondary or residual one. Fisher's hypothesis is identical with that of Fried et al. (1957), which has been discussed previously, with the additional conclusion that a renal cell sensitive to changes in oxygen status exerts the final control over REF production. Certainly the balance of evidence supports this contention (see Fisher, 1972, for review).

The possibility of a higher control over erythropoiesis involving the central nervous system has attracted the interest of several investigators. Most of the evidence which has been acquired involves increases in erythropoiesis following electrical stimulation of the hypothalamus (Feldman, Rachmilewitz & Izak, 1966; Medado, Izak & Feldman, 1967). In rabbits and monkeys, hypothalamic stimulation has been shown to produce elevated plasma EPO levels (Halvorsen, 1961, 1968; Mirand, Murphy & Bernardis, 1967) which were not abolished by hypophysectomy (Halvorsen, 1961; Mirand et al., 1967). In both rats and rabbits administration of high doses of atropine has been shown to abolish the reticulocyte response to hypothalamic stimulation (Medado et al., 1967; Segal, Izak & Feldman, 1971; Paulo, Roh & Fisher, 1972). The high doses of atropine which were used might have produced ganglion, as well as cholinergic, blockade, so that the relative importance of the sympathetic and parasympathetic systems is not clear. Atropine has also been reported to inhibit the rises in plasma EPO titres which followed hypoxia in rabbits (Paulo et al., 1971, 1972). Plasma EPO levels were not elevated by hypothalamic stimulation in rats (Segal et al., 1971) and prolonged stimulation was shown to produce haemolysis, evident in the peripheral circulation. Stimulation of the sympathetic

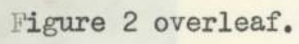


Figure 2 overleaf.

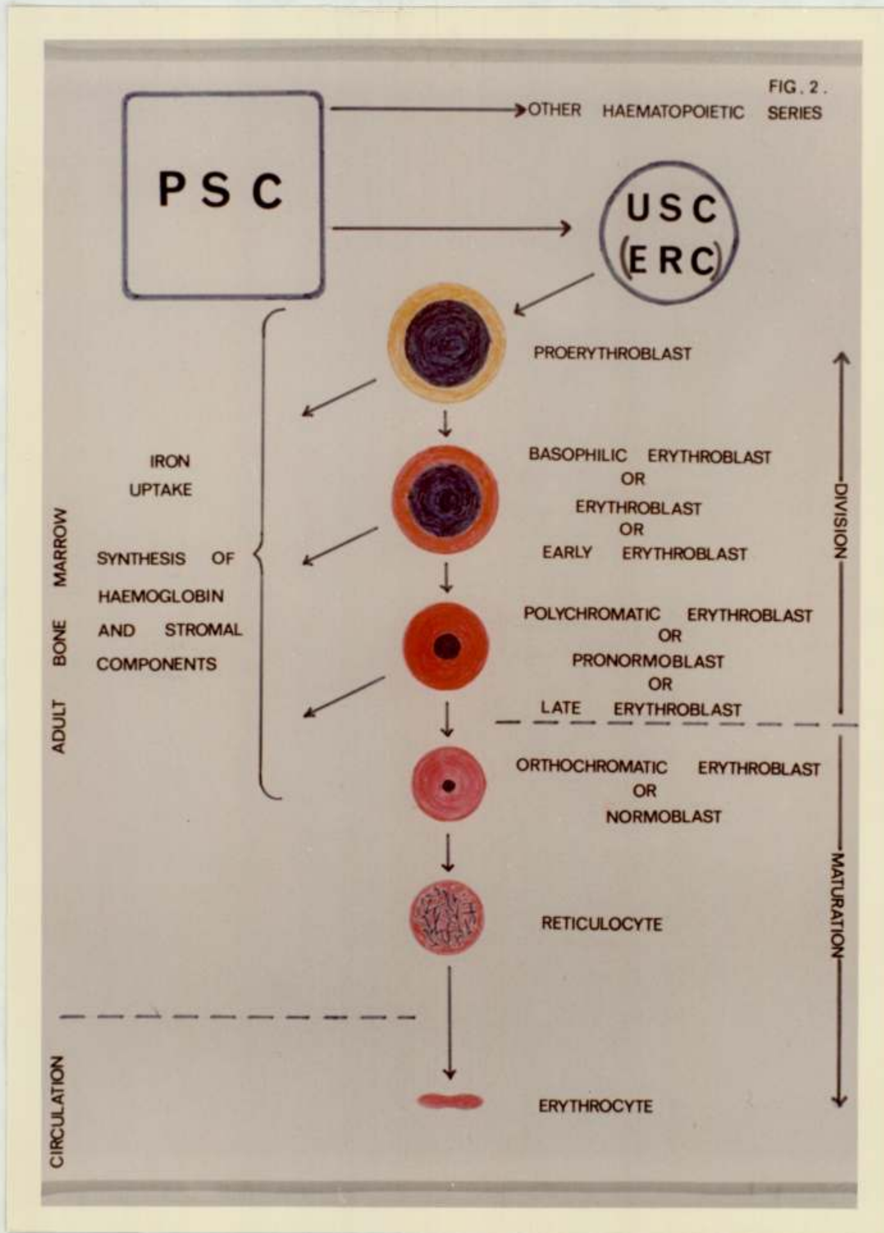


Figure 2: The erythroid series (the erythron). Alternative nomenclatures are indicated. PSC = pluripotential stem cell. USC (ERC) = unipotential stem cell (EPO responsive cell). For discussion, see text.

nerve trunks in rats has been found to increase reticulocyte release from bone marrow (Webber, DeFelice, Ferguson & Powell, 1970).

The significance of these scattered, and occasionally contradictory, observations is not established. However, it is clear that the major renal mechanism proposed by Gordon and Zanjani (Gordon et al., 1971) and Fisher (1969) may be partly modified by other factors which may have roles in the control of erythropoiesis.

2.8 The Erythron.

The evidence which has been presented above clearly indicates that in the rat, and in other mammalian species (Gordon & Zanjani, 1970), the formation of erythrocytes is principally regulated by a mechanism which involves changes in the elaboration of the hormone EPO. The mechanism of action of the hormone will now be considered in the light of the intricate series of differentiation and maturation phenomena which ultimately result in the genesis of the highly specialised erythrocyte. Collectively the several processes therefore comprise erythropoiesis.

The sum total of the cells in the erythroid series, i.e. the circulating elements and their precursors, has been termed the erythron (Boycott, 1929), a valuable concept which emphasises the functional unity of the whole. As mentioned previously, the major site of erythropoiesis in the adult mammal is the red bone marrow (Grant & Root, 1952). The bone marrow is a loose-knit, gelatinous and, to some extent, fatty tissue, enclosed within a rigid case of bone and possessing a distinctive vasculature (Weiss, 1970a).

The various stages of erythroid cell developement have been recognised on morphological, and, latterly, biochemical criteria (Marks & Kovach, 1966); these are depicted diagrammatically in Figure 2. There is no convention governing the nomenclature of these cells and

thus widely differing terminologies may be encountered; some of these alternatives are shown in Figure 2. The following terms will be used here for the erythropoietic sequence: pluripotential stem cell ⟶ unipotential stem cell or EPO-responsive cell (ERC) ⟶ proerythroblast ⟶ erythroblast ⟶ pronormoblast ⟶ normoblast ⟶ reticulocyte ⟶ erythrocyte. The brevity of these terms gives them advantages over others, and they can be considered no more trivial than names ascribed purely on the basis of reactions to certain histochemical techniques.

The development of mammalian erythroid cells involves a continuous progression from a primitive progenitor cell without detectable haemoglobin content to a mature, non-nucleated erythrocyte in which more than 95% of the total protein content is haemoglobin (Thorell, 1947). Proerythroblasts have a large nucleus : cytoplasm ratio, incorporate tritiated (^{3}H) thymidine into DNA rapidly and contain little or no haemoglobin; ribosome content decreases and haemoglobin content increases in the later stages of the erythroid series (Marks & Kovach, 1966). In man the proerythroblast has a diameter of 12μ and each successive stage has a reduced diameter with approximately 50% of the volume of its predecessor (Thorell, 1947) This progression is also thought to occur in the rat (Marks & Kovach, 1966).

The erythroid progression involves processes of differentiation, multiplication and maturation which normally occur in an orderly manner (Bond, Fliedner, Cronkite, Rubini & Robertson, 1959), although perturbations may occur. The proerythroblast, erythroblast and pronoroblast are capable of division but later stages are not (Grasso, Woodard & Swift, 1963), having lost the ability to synthesize DNA. The nucleus is extruded from the cell after the normoblast stage to yield the

reticulocyte (Pease, 1956). In man this may be retained within the marrow for 1-2 days before release into the circulation where it may retain its reticulum for a further 24 hours (Harris & Kellermeyer, 1970a) before becoming morphologically indistinguishable from other erythrocytes.

2.9 The Stem Cell Concept.

The erythroid series is classifiable as a renewing, or steady state, series (Messier & Leblond, 1960). Since the red cell count in rat blood is 8 - 10 x 10^9 cells/ml (Hunt, unpublished observations) and their circulating life-span is approximately 60 days (see Section 2.2), a 200g rat will require to replace some 10^9 erythrocytes each day, assuming a blood volume of 6ml/100g body weight. This naturally poses the question of the origin of the considerable numbers of erythroblasts necessary for this high level of production. The existence of an ultimate precursor, or stem cell, which has the dual capabilities of generating erythroblasts and maintaining its own numbers has been postulated. The alternative possibility that stem cells could be present at birth in numbers sufficient to generate all the erythroblasts required throughout life has been discounted (Lajtha, 1970).

The work of Jacobson and his colleagues (Jacobson et al., 1957) provided the initial evidence for the existence of a stem cell compartment from which the proerythroblast is derived. Transfusion-induced polycythemia was found to produce a virtual absence of erythroid elements in mouse bone marrow within a week, though subsequently the animals were capable of an erythropoietic response. More recent observations in a variety of species have established the validity of the stem cell concept. Thus:

(i) In dogs, Alpen and Cranmore (1959) found that proerythroblasts

labelled with ^{59}Fe became diluted by non-labelled proerythroblasts much sooner in haemorrhaged animals than in controls;

(ii) In rabbits, Erslev (1959) found that erythroblast proliferation could proceed in the absence of EPO and that proerythroblast formation was unaffected by the mitosis-arresting agent colchicine, thus indicating that their formation was by differentiation from a precursor rather than by proerythroblast replication; and

(iii) The spleens from polycythemic mice incubated with EPO developed proerythroblasts, though these were absent prior to incubation (Mohit & Sato, 1967).

These and other studies have indicated that in a variety of species EPO acts upon a primitive erythropoietin-responsive cell (ERC) to induce differentiation into the proerythroblast which further matures to give the other erythroblast stages. The ERC is in turn derived from an even more primitive "true" stem cell.

2.10 Pluripotential and Committed Stem Cells.

After the initial observation that splenic shielding during lethal X-irradiation enhanced murine survival (Jacobson, Marks, Gaston, Robson & Zirkle, 1949) it was reported that injections of cell suspensions of haemopoietic tissue into irradiated mice also resulted in decreased mortality (Jacobson, Marks & Gaston, 1954). These transplanted cells were found to repopulate the haemopoietic tissues of the irradiated hosts (Ford, Hamerton, Barnes & Loutit, 1956). Till & McCulloch (1961) injected dilute suspensions of nucleated haemopoietic cells (10^4 - 10^5 total) into lethally irradiated mice and observed discrete nodules, or colonies, of proliferating haemopoietic tissue in the recipients' spleens. The number of spleen colonies was directly proportional to the number of injected marrow cells. Since the identity of the cells

Figure 3 overleaf.

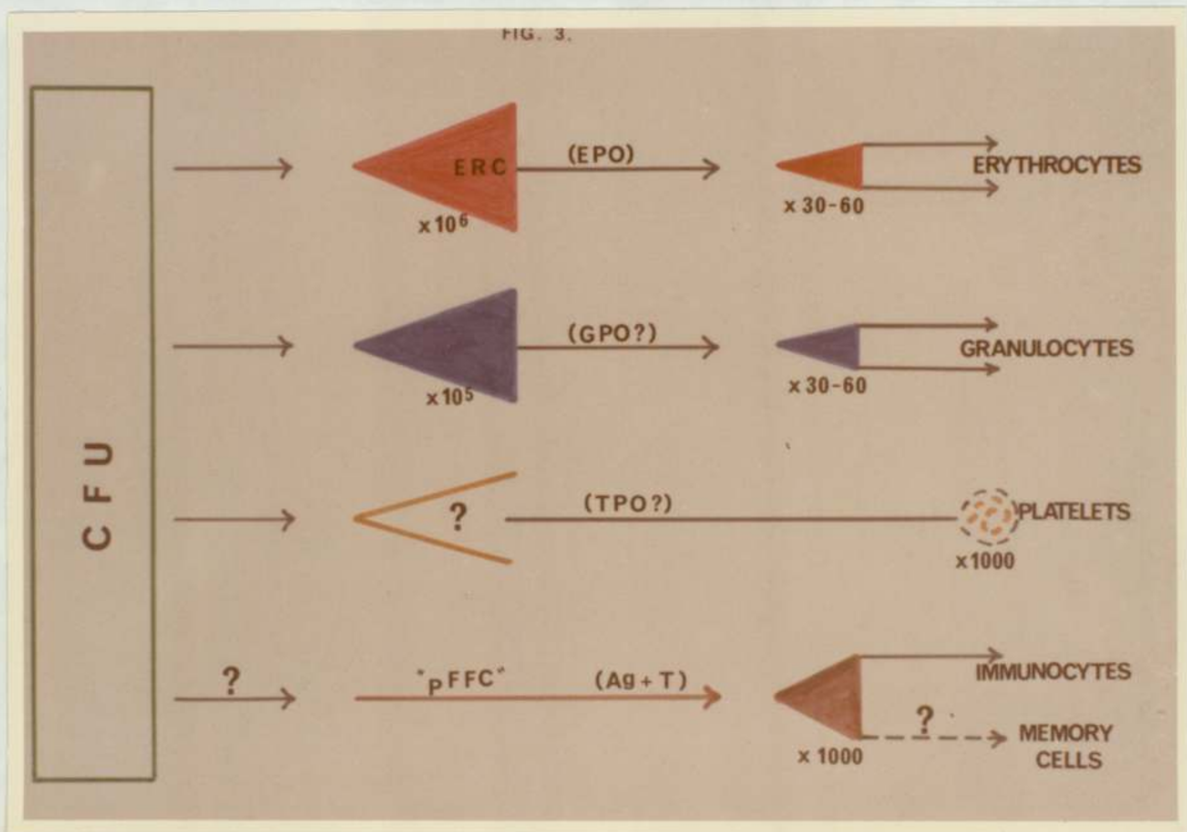


Figure 3: A model of the pluripotential haemopoietic stem cell and derived populations. Slightly modified from Lajtha, 1970. Nomenclature discussed in text.

giving rise to colonies was unknown, they were termed colony forming units (CFU) by Till & McCulloch. This extremely useful technique for the investigation of stem cell kinetics is known as the exogenous spleen colony-forming method; the endogenous method involves the re-population of irradiated haemopoietic tissues from healthy tissue (protected by lead shielding) within the animal.

Granulopoiesis, erythropoiesis and thrombopoiesis have all been observed in spleen colonies (Lewis & Trobaugh, 1964) and chromosome marker studies have demonstrated that the colonies are clonal, i.e. derived from single cells (Becker, McCulloch & Till, 1963). Thus these cells (CFUs) are pluripotential, i.e. capable of giving rise to at least three major haematopoietic series. A careful distinction must be drawn between the various definitions of CFU which are encountered in the literature; a variety of techniques e.g. exo- and endo-colonising spleen, erythropoietic repopulation or agar colony assays, show the properties of different types of stem cell with varying characteristics. However, the method which unequivocally demonstrates the pluripotential stem cell is the exocolonising spleen assay (Lajtha, 1970). Thus "CFU" in this discussion will refer to the "spleen exocolony forming unit". Chromosomal marker studies by Wu et al., (1967) have demonstrated that the CFU has pluripotential characteristics and the capacity to reproduce itself.

These considerations have led to the propounding by Lajtha (1970) of the composite model for the pluripotential stem cell and associated populations shown in Figure 3:

(a) The CFU is equivalent to the pluripotential stem cell. Less than 10% of this population are actively cycling at any one time (Becker, McCulloch, Siminovitch & Till, 1965; Lajtha, Pozzi, Schofield & Fox, 1969). This could indicate a mean turnover time in the mouse of approx-

imately 10 days. The stem cell is capable of giving rise to a number of differing cell populations; the first stage in the erythroid series is probably the morphologically unidentified ERC. The factors controlling the differentiation into the ERC are not understood.

(b) The ERC is a committed or unipotential cell population which is constantly cycling, even in the polycythemic mouse where there is no demand for erythropoiesis (Lajtha et al., 1969). Approximately 70% of the cycle is spent in DNA synthesis. The ERC has been shown by colonising studies to be capable of some 20 or more cycles, i.e. a multiplication factor of $x10^6$. The ERC represents the first stage in an irreversible pathway terminating in cells with a finite life-span. EPO acts upon the ERC to induce haemoglobin synthesis.

(c) The existence of a granulocyte precursor cell (GPC) is obvious, but it is not clear whether this is analogous to the ERC or identical with it, or even with the CFU. It is likely that there is a GPC population which undergoes a series of amplifying divisions in the same way as the ERC. However, the status of this population, and the possibility that a granulopoietin (GPO) acts upon it to induce differentiation into the granulocytic series, is not settled.

(d) Chromosome marker studies have demonstrated that megakaryocytes are ultimately derived from the CFU. The existence of a unipotential thromboid precursor analogous to the ERC (Ebbe & Stohlman, 1965; Morse & Stohlman, 1966) and a thrombopoietin (TPO) (Ebbe, 1968; Cooper, 1970) have been inferred but not demonstrated.

(e) It is likely that the CFU is also the origin of antibody-forming cells (plaque-forming cells, PFC). The precursors of the PFC have been designated potential focus-forming cells (pFFC), as a consequence of their ability to give rise to splenic foci in irradiated mice injected with spleen cell suspensions. The rate of turn-over of the pFFC

is small until stimulated by cells of thymic origin (Miller & Mitchell, 1967; Gregory & Lajtha, 1968; see Section 3.2 for full discussion).

Much of the above evidence has been derived from studies with hypertransfused or normal mice. However, there is thus far no evidence to show that the rat exhibits any significant divergence from the described patterns. Inferentially, since many of the murine observations have been confirmed in other mammalian species, including some in rats, the concept of the pluripotential stem cell may be applied to the albino rat.

2.11 Sites and Mechanisms of Action of EPO.

The concept that EPO acts upon the unipotential erythroid stem cell (ERC) has been mentioned previously. In addition to this differentiating action, EPO has been postulated to act upon erythroblasts to produce a range of effects which will be discussed below. The time course of the erythron's response to EPO in the plethoric mouse has been determined (Filmanowicz & Gurney, 1961; Orlic, Gordon & Rhodin, 1968). One day after the administration of EPO, heightened erythropoiesis was revealed by a peak in the number of proerythroblasts in the bone marrow, followed at approximately 2.5 and 3.5 days by similar increases in erythroblasts and reticulocytes.

The action of EPO upon the ERC in mice is postulated to be at the G_1 phase of the cell cycle (Hodgson, 1970). Experimental evidence for the nature of this activity is limited, but Hodgson (1970) has suggested that EPO acts as an inducing agent which causes gene derepression, resulting in the coding of mRNA specific for the production of elements characteristic of the erythroid series, e.g. haemoglobin. This author concedes, however, that the available evidence can not be interpreted strictly in terms of the action of the hormone upon its target ERC until that population has been isolated from other unipotential stem

cells (if they exist), pluripotential CFUs and other elements of the four haemopoietic series.

Since erythroblasts can be identified in a number of ways, the number of studies concerned with their responses to EPO has outweighed that on the actions of EPO on the ERC. Many of these experiments have been performed upon rat bone marrow cells isolated in vitro. EPO has been shown to increase RNA synthesis in these cells within 15 minutes; this action was abolished by actinomycin D, suggesting that this action of the hormone is upon DNA-dependent RNA synthetic processes in target cells (Krantz & Goldwasser, 1965). This observation was later extended and the formation of at least 6 types of RNA, including ribosomal, transfer and messenger types, has now been observed to be induced by EPO in rat bone marrow in vitro (Gross & Goldwasser, 1969). DNA synthesis increases following this early increase in RNA synthesis (Krantz & Jacobson, 1970b). Inhibition of DNA synthesis had little effect upon increases in RNA synthesis induced by EPO (Gross & Goldwasser, 1972), whereas the actinomycin D inhibition of RNA synthesis did prevent the increases in DNA (Gross & Goldwasser, 1970) and haemoglobin synthesis. It therefore seems likely that the induction of increased RNA synthesis is the primary step in the action of the hormone upon erythroblasts (Krantz, 1973). The EPO "message" is thought to be transmitted from cell membrane sites to the nucleus via a cytoplasmic intermediary. This has been demonstrated to be distinct from adenosine 3', 5'-monophosphate (Graber, Carrillo & Krantz, 1972; Krantz, 1973), which performs this role in many hormone/cell interactions (Robinson, Butcher & Sutherland, 1971).

These biochemical actions of EPO have a dual effect upon erythroblasts, which serves to accelerate their maturation time:

(i) EPO has been demonstrated to decrease cycle time in the proliferating erythroblasts of anaemic rats (Tarbutt, 1969), which may explain the increased mitotic index observed after EPO administration (Matoth & Kaufman, 1962; see General Methodology, Section 6.3 for rationale). DNA and haemoglobin synthesis are also increased by EPO (Powsner & Berman, 1967). In addition, the marrow transit time of erythroblasts in anaemic rats is decreased by EPO (Blackett, 1968; Tarbutt, 1969). The appearance of macrocytic reticulocytes and erythrocytes in the circulation of rats and mice (Blackett, 1968; Paul, Conkie & Burgos, 1973) after EPO treatment has been ascribed to a shortening or complete bypass of the normoblast maturation stage, and consequent release of immature reticulocytes from the bone marrow (Gordon & Rhodin, 1965; Krantz, 1973; Paul et al., 1973). It has also been suggested (Blackett, 1968) that EPO could increase the amplification factor achieved by erythroblast mitosis by introducing an extra division in the early erythroblastic stages.

(ii) EPO has long been known to directly stimulate the release of reticulocytes from isolated hind limbs of rats (Gordon, Lobue, Domfest & Cooper, 1962). This action is probably divorced from that which causes release of immature reticulocytes and the mechanism is unknown.

The sites of action of EPO in the rat are probably, therefore, both the ERC and the erythroblast elements of the erythroid series. Other possibilities are investigated later in the present study (Results, Chapter 2).

2.12 Other Factors Influencing Erythropoiesis.

Agents could be postulated to affect erythropoiesis by a number of different routes:

(i) by changing the oxygen supply/demand ratio of the tissues;

(ii) by direct actions on the erythropoietic tissues; or

(iii) by modifying EPO production.

A number of agents which fall into one or more of these categories have been considered previously in this Introduction. Further discussion will be chiefly confined to the hormones and ions which are relevant to the subject matter of the studies reported later.

The stimulatory action of cobalt upon erythropoiesis has been established for many years (Waltner & Waltner, 1929; Jacobson & Goldwasser, 1958). Early hypotheses suggested that cobalt exerted its effect by a direct histotoxic action on bone marrow, but this explanation was re-examined in the light of later observations upon the regulation of erythropoiesis by EPO. Cobalt was observed to increase circulating EPO levels in normal, fasted and hypophysectomised animals within 12h (Jacobson & Goldwasser, 1958; Rodgers, George & Fisher, 1972; Smith & Contrera, 1972). The ion was shown to increase cyclic AMP concentration within certain renal cells (Rodgers et al., 1972). The elevated intracellular concentration of the cyclic nucleotide are postulated to stimulate formation of the REF. In vitro studies have suggested that the cobalt-sensitive cells are located within the renal medulla in sheep (Chowdhury & Datta, 1973). Cobalt has also been reported to increase nucleated erythroid cell counts in dog bone marrow by a direct action (Fisher, Roh, Couch & Nightingale, 1964).

Hypophysectomy has been shown to inhibit erythropoiesis in both male and female rats (Crafts, 1941; Gordon, 1954; Crafts & Meineke, 1959; Bozzini, 1965). Posterior or intermediate lobe removal did not have this effect and therefore the response was dependent upon the anterior lobe (Van Dyke, Garcia, Simpson, Huff, Contopoulos & Evans, 1952). Several studies have led to the conclusion that the depression of erythropoiesis was secondary to an overall reduction in metabolic rate and therefore in oxygen demand/supply ratio (Fried et al., 1957; Crafts & Meineke, 1959). The effects of hypophysectomy on erythro-

poiesis could be reversed by administration of various combinations of hormones, e.g. thyroxine (Gordon, 1954; Crafts & Meineke, 1959).

Thyroidectomy or thyroid insufficiency produced an anaemia which was reversible by administration of thyroxine (Gordon, Kadow, Finkelstein & Charipper, 1946). This has been ascribed to a depression of metabolic rate and, consequently, oxygen demand/supply ratio (Jacobson, Goldwasser, Gurney, Fried & Plzak, 1959). Administration of thyroxine to intact rats stimulated erythropoiesis (Donati, Warnecke & Gallagher, 1964, 1966) but had no effect in nephrectomised animals (Carnevali, Lucarelli, Ferrari, Rizzoli, Parcelline & Butturini, 1968), suggesting that thyroxine acted via an increase in EPO production. This has been confirmed by the demonstration that thyroxine increased plasma levels of EPO (Peschle, Zanjani, Gidari, McLaurin & Gordon, 1971). However, it has not been unequivocally demonstrated that this action of thyroxine is dependent upon an increase in the oxygen demand/supply ratio. Thus the possibility of a direct action upon the REF forming cells in the kidney, or upon renal blood flow, cannot be discounted.

A number of conflicting observations on the possible roles of adrenal hormones in erythropoiesis make interpretation of the role of the adrenal gland in erythropoiesis difficult. Administration of adrenal corticoids has been reported to stimulate (Fruhman & Gordon, 1956; Fisher, 1958), inhibit (Glader, Rambach & Alt, 1968) or not affect (Morrison & Toepfer, 1967) erythropoiesis in rats. The action appeared to be dependent upon the dose administered and upon the endocrinological status of the experimental animal. It is therefore not clear whether the corticosteriods have any role in the maintenance of erythropoiesis under normal conditions. However, adrenal corticoids have been demonstrated to decrease leucocyte production (Gordon, 1959) and to inhibit the growth of thymus, mesenteric lymph nodes and

spleen (Dougherty, 1952; Santisteban & Dougherty, 1954; see also Section 3). Aldosterone injection has been shown to have little effect upon erythropoiesis (Mann, Donati & Gallagher, 1966; Zivny, Neuwirt & Borova, 1972; see also Results, Chapter 3), although this contention has been disputed (Cooper, Zanjani & Gordon, 1968). Noradrenaline infusion increased EPO production (Fisher, Samuels & Langston, 1968). This was postulated to be secondary to the constriction of afferent renal blood vessels and a consequent reduction in renal oxygen supply.

The role of androgens in erythropoiesis has been extensively studied. Castration was shown to produce a slight anaemia, which was reversible by androgen administration (Steinglass, Gordon & Charipper, 1941). Testosterone has been demonstrated to increase circulating EPO titres in rats (Gordon, Mirand, Wenig, Katz & Zanjani, 1968a; Gordon, Zanjani & McLaurin, 1968b) via an increase in REF production (Gordon _et al._,1968b). Although it did not prove possible to show correlations between metabolic rate and erythropoiesis after androgen treatment (Meineke & Crafts, 1968) the insensitivities of gross measurement of metabolic rate render the demonstration of such a relationship unlikely. As in the case of thyroxine, it is probably necessary to study the effects of the hormone on isolated or _in situ_ perfused kidneys to determine whether there is a direct component of its activity. These studies have not been reported in the literature. The possibility that androgens have a synergistic action with EPO upon target cells in marrow is unresolved (Krantz & Jacobson, 1970c).

Ovariectomy was shown to increase erythropoiesis in normal rats (Steinglass _et al._, 1941; Vollmer & Gordon, 1941). Administration of oestradiol has been shown to depress erythropoiesis in male rats (Dukes & Goldwasser, 1961) and also reduce the erythropoietic response to hypoxia in animals of that sex (Gordon _et al._, 1968b). The latter

authors have suggested that oestrogens may inhibit formation of erythropoietinogen, since REF levels and activity remained unaffected while EPO production was decreased. However, the doses used in their studies were grossly unphysiological and it seems most likely that the primary action of oestradiol is to inhibit the effect of EPO upon its target cells in the bone marrow (Dukes & Goldwasser, 1961). The stimulatory action of testosterone and inhibitory action of oestradiol on erythropoiesis may explain the common observation that red cell counts are higher in male rats than in female (Steinglass et al., 1941; Grant & Root, 1952).

Stimulatory effects on erythropoiesis have also been reported for a number of other hormones, the most relevant to the present study (Results, Chapter 3) being vasopressin (Jepson, McGarry & Lowenstein, 1968) and angiotensin II (Nakao, Shirakura, Azuma & Maekawa, 1967). However, those experiments were performed upon man and rabbit respectively; no evidence has been advanced to support the existence of an effect of either of these hormones on erythropoiesis in the rat.

The hormones involved with the control of calcium homeostasis and the status of the calcium ion itself have been demonstrated to have important roles in the control of cell division and erythropoiesis (Rixon, 1968; Perris, MacManus, Whitfield & Weiss, 1971; Perris & Whitfield, 1971). Since the areas of investigation in the present study have largely devolved from these and other observations (Perris, 1971; Whitfield, Rixon, MacManus & Balk, 1973c), the role of the calcium homeostatic system in the physiological control of haemopoiesis and lymphopoiesis will be discussed in detail later (Section 5).

2.13 Summary.

Circulating blood cells, though distinct both structurally and functionally, are ultimately derived from a common stem cell. This

cell has been designated the pluripotential stem cell and its properties may be demonstrated by observation of the CFU, with which it is probably synonymous, in the exocolonising spleen assay. A "committed" (unipotential) stem cell, derived from the pluripotential stem cell, has been demonstrated for the erythroid series in the bone marrow and it seems likely that equivalent populations exist in the granuloid and thromboid series. None of the stem cells are morphologically identifiable at present and studies of their natures and control systems have therefore been indirect.

The numbers of circulating blood cells are subject to control systems which have been delineated to varying degrees. While the factors which maintain granulocyte, platelet and lymphocyte numbers are imperfectly understood, the control of circulating red cell mass has been studied in some depth. The oxygen supply/demand ratio of tissues appears to constitute the fundamental stimulus for erythropoiesis. A renal cell is postulated to act as a sensor for perturbations in this ratio and to effect changes in the synthesis of an enzyme, the renal erythropoietic factor (REF) or erythrogenin, which acts upon a plasma substrate, of possible hepatic origin, to liberate the hormone erythropoietin (EPO). The primary action of this hormone is upon the unipotential erythroid stem cell (EPO responsive cell — ERC) to cause differentiation into a cell possessing recognisable erythroid characteristics. Direct actions of EPO upon maturing erythroid elements have also been demonstrated.

Although a number of hormones have been shown to affect erythropoietic activity it is clear that in general they do not constitute physiologically significant control systems but merely impinge upon the EPO/ bone marrow axis. However, important exceptions to this generalisation are the hormones of the calcium homeostatic system which

act as modulators of the rate of proliferation of cells in the bone marrow.

Section 3. The Thymus.

3.1 General Considerations.

To consider the thymus independently of haemopoiesis in this Introduction is a procedure open to criticism on a very basic level, for, in mice at least, thymocytes are ultimately derived from the pluripotential stem cells of bone marrow (Ford & Micklem, 1963; Miller & Osaba, 1967; Metcalf, 1970). However, this distinction has been made here, partly on the grounds of convenience and partly because of the characteristics of lymphopoiesis which distinguish it from the other haemopoietic series. In particular, cell proliferation in the erythroid, granuloid ~~and~~ and thromboid series is conducted exclusively in the bone marrow in the adult rat, whereas lymphopoietic divisions occur in various lymphoid tissues (spleen, lymph nodes, Peyer's patches, thymus) following the "seeding" of precursors from bone marrow (Metcalf, 1970).

Since the large and medium thymocytes (in mice) have a short cycle time of only some 6 - 8 hours (Metcalf & Wiadrowski, 1966) the thymus is a suitable tissue for the study of hormonal and ionic influences upon mitosis, both *in vivo* and *in vitro*. Further, the role of the thymus in the immune response (which is still incompletely understood) may indicate physiological significance for these influences in the response of the animal to antigenic stimuli. The majority of the work which has, to some extent, elucidated the role of the thymus in host defence mechansisms has been performed in mouse and guinea-pig. However, as noted by Weir (1971a):

"....once components of the immune system appear in evolution they are maintained with a remarkable constancy both at the molecular and functional level."

Thus the following observations may be assumed to broadly apply to most mammals and therefore to the rat.

3.2 Role of the Thymus in the Immune Response.

Although an extensive review of immunological processes is beyond the scope of the present Introduction it would be inappropriate to discuss the thymus in any context other than its physiological role. A brief summary of the characteristics of immune processes is therefore given here.

Foreign material entering the mammalian body encounters a number of innate, non-specific defence mechanisms (summarised by Weir, 1971b), such as physical barriers (skin and mucous membranes), external secretions (sweat, mucous and sebaceous fluid), internal tissue fluids (containing lysozymes or basic polypeptides) and finally phagocytosis (by micro- or macro-phages). The macrophages also play a role in the second line of defence to foreign particles, the immunological response.

The immunological response is characterised by the formation of substances known as antibodies, each of which is largely specific for one particular antigen (a chemical substance, usually foreign to the host, which stimulates the immune response). The response takes two forms which usually develop in parallel (Roitt, 1971):

(i) The production of humoral antibodies, which are globulins and which combine specifically with the antigen which stimulates their production. The actions of these immunoglobulins are various, e.g. producing agglutination and cell lysis and stimulating phagocytic activity; and

(ii) The production of lymphocytes carrying specific antibodies on their surfaces. These cells usually produce lysis of foreign (or certain tumour) cells by direct contact.

The latter phenomenon is dependent upon the presence of the thymus in foetal and neonatal life. The result of thymectomy of neonatal rats (Arnason, Jankovic, Waksman & Wennerstein, 1962) and mice (Miller, 1962a)

was a dramatic reduction in the capability of the animal to produce this cell-mediated immune response. Humoral antibody production was not quantitatively affected by thymectomy (Humphrey, Parrott & East, 1964) but some deficiencies in the humoral immune response to certain antigens have been noted (Papermaster, Kalmasso, Martinez & Good, 1962).

The thymus thus appears to be more intimately concerned with the development of the cell-mediated immune response than with the production of humoral antibody. Thus the discussion of the humoral immune response will be abbreviated here. It must be noted, however, that, as discussed below, co-operative interactions between the two systems undoubtedly occur and it is therefore unsound to completely divorce considerations of their respective characteristics.

The existence of a "secondary" immune response, which develops more quickly and is of greater magnitude than the "primary" response to antigen, suggests the existence of "memory cells" within the lymphoid populations. These can be envisaged as cells which are "programmed" or "coded" by the initial exposure to an antigen and can respond more quickly on subsequent exposure. The morphology of these cells has not been definitely established and they are therefore best designated (Metcalf, 1970) "antigen-sensitive cells (ASCs)". These cells must be present in spleen and lymph nodes, which respond to antigenic stimulus, and absent in bone marrow and thymus, which do not (Kennedy, Siminovitch, Till & McCullogh, 1965; Miller & Mitchell, 1967). Antigen stimulation probably causes differentiation and proliferation of these ASCs, ultimately resulting in the formation of plasma cells and possibly small lymphocytes (Metcalf, 1970). The plasma cells synthesize humoral antibody (Nossal & Malcela, 1962). In the chicken a central lymphoid organ, the Bursa of Fabricius, exerts a controlling influence over the development of cells capable of synthesizing antibodies (Glick, Chang & Jaap,

1956; Szenberg & Warner, 1964). This control may be humoral in nature (Glick, 1960). Several lymphoid organs have been suggested as mammalian analogues of the Bursa of Fabricius, including the appendix in the rabbit (Sutherland, Archer & Good, 1964) and the Peyer's patches and tonsils in man (Peterson, Cooper & Good, 1965; Cooper, Perey, McKneally, Gabrielsen, Sutherland & Good, 1966). The lymphocyte precursors of the plasma cell series have been designated B-lymphocytes (i.e. Bursa dependent) and those of the antibody-carrying cells T-lymphocytes (i.e. thymus dependent). Despite the functional difference between T- and B-lymphocytes it is clear that areas of co-operation between them exist. For example, it has been shown that co-injection of T- and B-lymphocytes is much more effective in stimulating humoral antibody production than is injection of B-lymphocytes alone (Claman, Chaperon & Triplett, 1966). This observation may help to explain the inhibition (noted above) of some humoral immune responses by neonatal thymectomy (Papermaster _et al._ 1962). Thus, although the thymus is principally involved in the regulation of cell-mediated immunity it also has an important role in the humoral antibody response to certain antigens.

The lymphocyte population of the thymus is broadly classifiable into three categories: small (constituting approximately 90% of the total thymocyte population), medium (approximately 10%) and large (1%) thymocytes (Metcalf & Wiadrowski, 1966). None of these categories have been observed to proliferate in response to antigenic stimuli (Metcalf, 1961) or exhibit reduced proliferation in germfree conditions (Wilson, Bealmear & Sobonya, 1965) as do lymphocytes in lymph nodes and the spleen. Despite this insensitivity to antigenic stimulation it is appealing to postulate that the thymus could export

large numbers of lymphocytes to the peripheral lymphoid tissue where they would become capable of entering the cell-mediated immune response. The attraction of this hypothesis derives from the observation that the thymus normally sustains a high degree of mitotic activity (Metcalf & Wiadrowski, 1966). Note that mitosis is limited to the large and medium thymocyte (lymphoblast) population (Metcalf & Wiadrowski, 1966). Supporting the concept that the thymus seeds peripheral lymphoid tissues, but only to a small extent, are a number of experimental observations:

(i) Thymectomy in young adult mice causes a small atrophy of peripheral lymphoid tissue, though the development of this atrophy is slow (Metcalf, 1960; Metcalf & Brumby, 1966);

(ii) Thymectomy in neonatal mice produces a more profound lymphoid atrophy (Miller, 1962a); and

(iii) Radioactive labelling shows that small numbers of thymocytes from thymus grafts migrate to certain areas of lymph nodes and the spleen (Nossal, 1964).

The seeding role of the thymus is thus smaller than might be predicted on the basis of its high intrinsic mitotic activity. The few thymocytes which are "exported" to other lymphoid tissues might proliferate there, under antigenic stimulation, and influence the production of immunologically competent cells. However, several studies have demonstrated that the thymus has a major role in the immune response which is probably independent of thymocyte production.

Thymus grafts in small-pore diffusion chambers have been shown to preserve the immunoreactivity of neonatally thymectomized mice to a number of antigenic stimuli and to reduce lymphoid atrophy (Osoba & Miller, 1963, 1964). Injections of thymus extracts had similar

effects (Trainin & Linker-Israeli, 1967) and were also demonstrated to increase DNA synthesis in lymph nodes (Klein, Goldstein & White, 1965). This humoral factor is probably produced by epithelial or reticular thymus cells and not by thymocytes (Metcalf, 1956; Osoba & Miller, 1964). The principle site of action of the substance appears to be the peripheral lymphoid tissue. Note that this factor is probable distinct from that which produces proliferation of the bone marrow precursors of B-lymphocytes (Miller & Mitchell, 1967; Gregory & Lajtha, 1968; Section 2.10). This latter process is probably dependent upon the activity of thymus "nurse cells", which interact with the potential focus-forming cells (see Section 2.10).

It therefore seems that the thymus is involved in the generation of the immune response in two ways:

(i) A small proportion of thymocytes may be exported to peripheral lymphoid tissues and to the bone marrow where they influence the production of cells capable of antibody synthesis; and

(ii) A humoral factor, produced by non-lymphocytic thymus cells, acts upon peripheral lymphoid cells to regulate the genesis of cells capable of effecting the cell-mediated immune response.

3.3 Regulation of Thymocyte Proliferation.

As mentioned above (Sections 3.1 and 3.2), with special reference to the mouse, the thymus exhibits a high level of mitotic activity in the large and medium thymocyte populations (Metcalf & Wiadrowski, 1966). This is also observed in the rat (Craddock, Nakai, Fukuta & Vanslager, 1964; Perris & Whitfield, 1967; Perris, Whitfield & Rixon, 1967; Perris, Whitfield & Tölg, 1968; Perris, Weiss & Whitfield, 1970; Rixon, Whitfield & MacManus, 1970; Results, Chapters 1 and 6). A

number of studies in the rat have indicated that the small thymic lymphocytes, which are themselves mitotically inert, are derived from repeated divisions of the larger lymphoblasts (Sainte-Marie & Leblond, 1958, 1965; Craddock et al., 1964). The turnover time for 95% of the total small thymocyte population has been estimated at 3-4 days in mice (Metcalf & Wiadrowski, 1966) and rats (Craddock et al., 1964) by radioactive labelling studies. This intensive rate of proliferation poses three problems:

(i) The nature of the precursor population which can generate the cells which maintain this activity, without exhausting its own numbers;

(ii) the physiological role and fate of the small thymocytes which are the terminal population in the thymus; and

(iii) the nature of the control system which maintains thymocyte proliferation at its normal level and which also regulates changes from this steady rate.

The sequence large ⟶ medium ⟶ small thymocyte is biased towards production of the latter, with a consequent reduction in large thymocyte numbers (Metcalf, 1966a). Thus it seems clear that large thymocytes must ultimately be replenished from a precursor pool which has the characteristics of a stem cell population. Although some evidence suggests that large thymocytes may be derived from thymus epithelial cells (reviewed by Miller & Osoba, 1967) it is clear that the ultimate precursors of thymocytes are chiefly of bone marrow origin. The use of chromosome markers (in mice) has unequivocally demonstrated that: precursors of thymic lymphoblasts were exchanged in the cross-circulation of parabionts (Harris, Ford, Barnes & Evans, 1964); thymus grafts in thymectomised recipients were repopulated by host cells (Miller, 1964); thymuses in irradiated hosts were repopulated by bone marrow, and not by lymph-node, cells when they were administered sim-

ultaneously or singly (Ford & Micklem, 1963). The identity of these repopulating cells from the bone marrow is still obscure.

The intense mitotic activity of large and medium thymocytes takes place almost exclusively in the thymus cortex (Kindred, 1955; Sainte-Marie & Leblond, 1958, 1965). Medullary thymocytes exhibit different properties, including a virtual absence of mitotic activity (Metcalf, 1970), a morphology characteristic of peripheral lymphocytes (Murray, Murray & Pizzo, 1965) and decreased radiosensitivity (Trowell, 1961). Aut oradiography has indicated that the medullary thymocytes may comprise the 5% fraction of the small thymocytes which does not turn over every 3-4 days (Matsuyama, Wiadrowski, & Metcalf, 1966). These differences might indicate that medullary and cortical thymocytes are completely separate populations or that the medullary population is a more mature form of the cortical thymocyte population (Miller & Osoba, 1967). The similarities between medullary thymocytes and peripheral lymphocytes suggests that the former cells might emigrate from the thymus to seed lymph nodes and spleen. The use of chromosomally marked thymus grafts in mice demonstrated that small numbers of thymocytes did seed into the peripheral lymphoid tissues (Miller, 1962b; Miller, Osoba & Dukor, 1965) and that these numbers were greatly increased after antigenic stimulation (Miller, DeBurgh & Grant, 1965). However at least some of the seeding cells are likely to be of cortical origin, for ^{3}H-thymidine labelled cells have been shown to appear in peripheral lymphoid tissues after direct injection of the isotope into the thymus (Nossal, 1964) and medullary thymocytes are rarely observed to become labelled in this way (Matsuyama _et al._, 1966). It must be noted that these two observations were made in different species, namely guinea-pig and mouse respectively.

The studies described above obviously do not

account for the high level of mitotic activity present in cortical thymocytes. The fate of the cortical small thymocytes remained obscure until the work of Metcalf (1966a). Adult mice were given up to 50 thymic transplants and, one month later, were injected with ^{3}H-thymidine. The percentagesof labelled lymphocytes (up to 90%) in the thymus grafts and in peripheral lymphoid tissue were no higher than in the corresponding tissues in ungrafted control animals. Thus although ample numbers of thymocytes were potentially available the peripheral organs did not appear more noticeably seeded. The inescapable conclusion is that if emigration from the thymus occurs it is on a very small scale and that most of the cortical lymphycytes must die in situ. Thus although the fate of the small thymocytes is established, the physiological significance of their continual production and destruction, which is sustained into adult life, remains obscure. Certainly the presence of the thymus is necessary for the full expression of immune competence in adult life (Metcalf & Brumby, 1966) but it is clear that the presence of the organ during neonatal life is more crucial (Miller, 1962a). The relative importance of the humoral and seeding contributions of the thymus to the immune response is not fully understood, although the absence of the former faculty is demonstrably more disadvantageous to the animal. However, on the grounds of empiricist logic it seems unlikely that the maintenance of a pool of rapidly proliferating cells by the animal can be of no physiological importance.

The level of thymocyte proliferation is influenced by factors intrinsic and extrinsic to the thymus (Metcalf, 1970). The majority of the evidence for the existence of an intrinsic control factor has been derived from thymus grafting experiments in mice. Thymus grafts from donors of particular ages were shown to exhibit involution at the same age in hosts of various ages, strains and sexes (Metcalf, Sparrow,

Nakamura & Ishidate, 1961). Graft size and thymocyte mitotic activity were similarly independent of the host environment (Metcalf et al., 1961; Metcalf, 1962). The epithelial and reticuloendothelial "framework" of the organ appeared to constitute the intrinsic control centre of the thymus (Metcalf et al., 1961; Metcalf, 1962; Metcalf, 1970).

The conclusions of Metcalf and his colleagues must be examined in view of the well-established influences of extrathymic factors upon thymocyte proliferation. For example, adrenal corticosteroids and sex steroids have long been known to be inhibitors of thymopoiesis or to be actively lymphocytolytic (Dougherty, 1952; Santisteban, 1960a, b; Dougherty, Berliner, Scheebeli & Berliner, 1964) and thyroxine and growth hormone injections have been shown to increase thymic weight (Dougherty, 1952; Gyllenstein, 1962). In addition, a number of hormonal and ionic influences upon thymus mitotic activity in vivo have been demonstrated by Whitfield and his colleagues in rats:

(i) Injections of calcium and magnesium ions stimulated thymocyte proliferation (Perris et al., 1967);

(ii) During rapid growth in the young animal, thymocyte mitotic activity paralleled the concentration of ionised calcium in the plasma (Perris et al., 1968); and

(iii) Parathyroidectomy produced acute thymic involution by severely depressing thymocyte proliferation (Perris et al., 1970; Results, Chapters 1 and 6).

The observations of Metcalf in thymic grafting experiments could be reconciled with the above phenomena in a number of ways:

(i) The importance of the intrinsic factor could be greater in the mouse than in rat or guinea-pig;

(ii) Thymic grafts in thymectomised hosts could behave differently to thymuses in situ;

(iii) The endocrinological status and the ionic constitution of tissue fluids could have remained remarkably constant in the graft recipients; (iv) The extrinsic factors described above could act via the thymic epithelial cytoreticulum which might modify their actions differently under various conditions.

It is very difficult to explain why thymic grafts should react similarly to a vareity of endocrinological environments. In particular, the seeming independence of thymocyte generation from the sex of the host is difficult to understand. Furthermore, the _in vivo_ influences of calcium and magnesium ions and several hormones upon thymocyte proliferation have also been demonstrated _in vitro_ (e.g. Whitfield & Youdale, 1966; MacManus & Whitfield, 1969a, b; Whitfield, Perris & Youdale, 1969; Whitfield, Rixon & Youdale, 1969; Whitfield, MacManus & Gillan, 1970; Whitfield, MacManus & Rixon, 1970; MacManus, Whitfield & Youdale, 1971; Results, Chapter 6) and are therefore not mediated by epithelial cytoreticular cells, although it is possible that very dilute extracts of these cells could have appeared in the incubation media in the above studies. The most likely role for the intrinsic thymus factor is the regulation of the numbers of primitive (large) thymocytes available for subsequent generation of small thymocytes (Metcalf, 1970). The action of the calcium ion in this tissue is almost certainly to be either to increase recruitment of cells from the G_0 (resting) phase of the cell cycle (see General Methodology, Section 6.3) into DNA synthesis or to stimulate DNA synthesis in cells near the G_1/S boundary of the cycle (see Section 5.4). The respective contributions of the intrinsic thymus factor and the extracellular calcium environment to the maintenance of thymocyte proliferation might best be delineated by the culture of thymocytes _in vivo_ in diffusion chambers

in thymectomised animals. These studies have not been undertaken to date. Cell viability would be a problem in this experimental design.

The availability of stem cells from the bone marrow is likely to be a limiting factor on thymocyte proliferation unless compensatory mechanisms exist to modify the numbers of primitive thymocytes derived from varying stem cell populations. Age-dependent thymic involution does not appear to be a result of reduced seeding of the thymus by the bone marrow (Metcalf, 1965). Since the morphological identity of the bone marrow-derived stem cells has not been established it has proven impossible in most situations to determine whether observed perturbations in thymocyte proliferation are the result of changes in their availability.

In summary, it must be concluded that the maintenance of small thymocyte production is a balance between the proliferation of large and medium thymocytes and the loss of the progeny by cell death or, to a far lesser extent, emigration. Thymocyte proliferation is a balance between stimulatory influences (of both intrinsic and extrinsic factors) and inhibitory influences (hormonal in nature, and also possibly due to the altered availability of incoming stem cells from the bone marrow). The intrinsic stimulus is dependent upon thymic epithelial cytoreticular cells and the major extrinsic factor is probably the extracellular calcium ion concentration. In conclusion it should be noted that the thymus does not exhibit a "cell homeostasis" control mechanism analogous to that of the erythroid compartment. There appears to be no "feedback" control upon the rate of production of small thymocytes. Thymuses grafted in multiples of up to 50 achieve the same size as ungrafted thymuses (Metcalf, 1963a). This is in contrast to multiple spleen grafts which appear to exhibit the characteristics of a tissue under a feedback control (Metcalf, 1963b, 1964a). However, the thymus regenerates after cortisone-induced involution (Ishidate & Metcalf, 1963)

though not after thymectomy (Metcalf, 1964b).

It must be concluded that many aspects of thymocyte proliferation are poorly understood. The controlling influences acting upon the production of small thymocytes exhibit some unusual features and there is as yet no satisfactory explanation for the established pattern of small thymocyte production and death _in situ_.

3.4 Influence of the Thymus upon Haemopoiesis.

A small body of evidence has accumulated which suggests that the thymus may influence the production of other circulating blood cells in addition to lymphocytes. Since this evidence formed part of the rationale for some of the experiments reported later (Results, Chapter 6) it will be considered in the Introduction and Discussion of those studies.

3.5 Summary.

The thymus is a lymphoid organ which differs in many respects from the peripheral lymphoid tissues (spleen, lymph nodes, Peyer's patches, tonsils, etc.). Thymectomy in neonatal and, to a lesser extent, young adult rodents has demonstrated the importance of this organ in three areas:

(i) the maintenance of peripheral lymphoid tissue mass;

(ii) the physiological competence to produce humoral antibodies in response to certain, but not all, antigens; and

(iii) the maintenance of competence to elicit a cell-mediated immune response to a wide range of antigenic stimuli.

Production of thymocytes, a small number of which may seed peripheral lymphoid tissue, is probably necessary for the maintenance of a full range of humoral antibody response to antigens. The "exported" cells are probably a mixture of cortical and medullary thymocytes, which show distinct biochemical differences.

Cell-mediated immunocompetence is regulated by a humoral factor which is secreted by the epithelial "framework" of the thymus. The humoral factor probably acts upon peripheral lymphoid tissue to control the generation of antigen-sensitive cells.

Small thymocytes are produced by repeated divisions of large and medium thymocytes in the thymus cortex. Mitotic activity in the thymus proceeds at a consistently high rate. There is as yet no convincing explanation of this phenomenon. Small thymocytes are produced in large numbers but 95% of these die _in situ_ after 3-4 days.

The control of thymocyte proliferation appears to reside in an interplay of several factors:

(i) the availability of incoming stem cells of bone marrow origin, which give rise to the more primitive (large) thymocytes;

(ii) several hormonal influences, both stimulatory and inhibitory;

(iii) an intrinsic factor, or factors, produced by the thymic epithelial cytoreticulum; and

(iv) the most important extrinsic factor, which is the extracellular calcium ion concentration.

Although many facets of thymic physiology have been studied empirically, major questions remain unanswered. In particular, the reason for the sustained production of large numbers of small thymocytes, almost all of which are short-lived and do not leave the organ, is still obscure.

Section 4. The Calcium Homeostatic System.

4.1 General Considerations.

A number of situations have previously been mentioned (Section 2.12, p. 30 and Section 3.3, pp. 42 - 43) in which mitotic activity in bone marrow and thymus has been demonstrated to depend upon the extracellular status of the calcium ion. These situations include several which have crucial physiological significance for the normal animal (see Perris, 1971; Section 5). These situations will be further elaborated in Section 5 of this Introduction, but it is clear on the basis of the evidence already presented that the calcium ion has an important role in the physiological control of mitotic activity in at least two tissues (bone marrow and thymus). The status of the calcium ion in biological fluids is determined by the calcium homeostatic system, which is in fact an interplay of several "systems" or factors. Knowledge of the actions of these factors which control calcium homeostasis is obviously essential for the elucidation of the mechanisms by which cell proliferation in certain tissues is regulated.

It should be noted that the ionised form of calcium is physiologically active and that the concept of total calcium in body fluids is to some extent meaningless. However, the physicochemical properties of biological fluids which may effect the ionised fraction of calcium, e.g. temperature, protein content and pH (see General Methodology, Section 5.3 for detailed discussion), are themselves subject to homeostatic controls. Thus ionised calcium is often observed to vary as a function of the total calcium concentration in biological solutions. In general, however, the calcium homeostatic system is essentially one which regulates the levels of the ionised fraction of calcium in the body.

In addition to its role in the control of tissue proliferation (Section 5), the calcium ion is indispensable in a number of physiological processes:

(i) the formation and maintenance of the skeleton;

(ii) the regulation of membrane permeability, including an important function in neuromuscular excitability;

(iii) muscle contraction;

(iv) the activation of a range of enzyme systems;

(v) blood coagulation;

(vi) spindle formation during mitosis;

(vii) the formation of intercellular "cement", and therefore the maintenance of capillary permeability;

(viii) the synthesis and release of acetylcholine and other neurotransmitters; and

(ix) the production of milk.

The elucidation of the components of the calcium homeostatic system has perhaps suffered in the past because of neglect of the consideration of the physiological significance of the calcium ion in the above processes. With increased understanding of the basic phenomena, however, a large body of studies have been carried out which attempt to relate calcium homeostasis to calcium ion function (e.g. Kenny, 1962; Jowsey, 1967; Gray & Munson, 1969; Talmage, 1969; Lewis, Rafferty, Shelley & Robinson, 1971; Perris, 1971; Philippo, Lawrence, Bruce & Donaldson, 1972; Swaminathan, Bates & Care, 1972; Whitfield _et al._, 1973c).

Only some 1% of the calcium of the body is present in the circulation and soft tissues, the remainder being deposited in the skeleton (Catt, 1970). This important fraction is subject to regulation by

three hormones, parathyroid hormone (PTH), calcitonin (CT) and (the active metabolites of) vitamin D. This control is exerted by the integration of effects upon calcium absorption, calcium and phosphorus excretion and, most importantly, the turnover of bone mineral. PTH administration produces an increase in blood calcium levels (Rasmussen, Armaud & Hawker, 1964), CT produces depression of blood calcium (Hirsch, Voelkel & Munson, 1964) and vitamin D is essential for the actions of PTH upon bone and gut (Harrison, Harrison & Park, 1958; Harrison & Harrison, 1961). Since plasma calcium levels are maintained, albeit at a much reduced level, in both parathyroidectomised (PTX) and thyroparathyroidectomised (TPTX) animals (McLean, 1957) it is clear that only a portion of mobilisable calcium is under the control of these hormones. This phenomenon is discussed in the following section (4.2); subsequent sections (4.3, 4.4 and 4.5) consider the three hormones individually, as far as is practical.

4.2 Calcium Homeostasis in Hormonally-Deprived Animals.

Exchange of calcium between bone and plasma has been observed in PTX rats (Talmage, Kraintz & Kraintz, 1952), which have a plasma calcium concentration of only some 5-7 mg/100ml (c.f. Results, Chapters 1, 2 and 3). In addition, calcium has been removed from PTX animals by intraperitoneal lavage while blood calcium concentration remained steady (Talmage & Elliott, 1956; Comar, cited by McLean, 1957). Thus it seems that calcium may move between blood and labile bone mineral deposits even in the absence of PTH. Observations _in vitro_ by Talmage (1967b) strongly suggested that plasma is supersaturated with respect to bone mineral, for calcium was taken up by bone incubated in normal plasma. This "supersaturation with respect to bone" of plasma (Neuman & Neuman, 1958) is supported by the observed decrease of free calcium after PTX. PTH is postulated to oppose this tendency by resorbing

calcium from bone (Talmage, 1962). The steady plasma calcium levels which are eventually attained after parathyroid excision presumably reflect the equilibrium point of plasma and bone mineral. The tendency of plasma calcium to move into bone implies that the major calcium homeostatic problem of mammals is the prevention of hypocalcaemia. This suggests that PTH will normally be of greater importance than CT in the maintenance of plasma calcium levels within desirable limits in the intact animal.

Copp (1964) observed that deposition of calcium in bone increased when blood calcium levels rose and decreased when they fell. Thus after removal of the parathyroids calcium is deposited in bone at an ever-decreasing rate until plasma and bone are in equilibrium with respect to their calcium content. The movement of calcium between blood and labile mineral proceeds in both directions at equal rates until the introduction of PTH or alterations in blood calcium levels (McLean, 1957). If blood calcium concentration is artificially raised or lowered, the equilibrium with "labile" bone will become weighted towards or away from bone, respectively (Copp, 1964). Superimposed upon this crude control is the regulating effect of PTH (see below). It is considered that calcium exchange in aparathyroid animals occurs between blood and the labile fraction mentioned above, whereas PTH-induced bone resorption is from "stable" bone mineral (McLean, 1957; Elliott & Talmage, 1958).

4.3 Parathyroid Hormone (PTH).

The role of the parathyroid glands in the maintenance of calcium ion concentration in tissue fluids was first postulated 65 years ago (McCallum & Voegtlin, 1909). The preparation of a parathyroid extract capable of elevating plasma calcium levels in normal and PTX dogs was

accomplished by Hanson (1924) and Collip (1925) and the essentially pure hormone was isolated in 1959 (Aurbach, 1959; Rasmussen & Craig, 1959).

Administration of PTH to rats produces an initial hypocalcaemia of short duration (Parsons & Robinson, 1971), followed by a sustained increase in plasma calcium concentration and a decrease in plasma phosphate concentration. PTH administration in PTX rats produced a rapid elevation of plasma calcium which persisted for up to 30h (Rasmussen _et al._, 1964). The administration of actinomycin-D prior to PTH did not prevent the initial rise in plasma calcium but reduced the duration of the hypercalcaemia to 6h. This action of actinomycin-D has been confirmed in bone cultures (Raisz & Neimann, 1967) and therefore suggests that RNA synthesis is a prerequisite for the sustained action of PTH upon its major target organ, the skeleton.

Although PTH has been demonstrated to increase calcium absorption from gut (Rasmussen, 1959), stimulate calcium reabsorption from renal tubules (Talmage & Kraintz, 1954; Kleeman, Rockney & Maxwell, 1958) and promote renal phosphate excretion (Talmage & Kraintz, 1954) there is no doubt that the primary action of PTH is to stimulate calcium resorption from bone (c.f. McLean, 1957). The actions upon gut and bone are dependent upon the presence of vitamin D (Harrison _et al._, 1958; Harrison & Harrison, 1961; see also Section 4.5). Three types of bone cell have been implicated at various times in the bone resorptive effect:

(i) Osteoblasts, the "bone-forming" cells (Fell, 1932) which are seen in large numbers at the sites of bone deposition in the rat (McLean & Urist, 1968);

(ii) Osteocytes, which appear to be osteoblasts which have become slightly modified after enclosure in newly deposited bone. Some may

synthesize parts of the bone matrix (Young, 1962), and they may also play a role in calcium exchange with plasma (Talmage, 1967a; Nichols, 1970), although some criticisms of this view have been expressed (Hancox, 1972); and

(iii) Osteoclasts which have classically been considered to mediate bone/plasma calcium exchange, since they are concentrated in areas of active bone resorption (Scott & Pease, 1956; Hancox, 1972).

As inferred above, the mechanism of action of PTH upon bone is the subject of some dispute. The hypotheses suggested by Talmage (1967a) and Belanger (Belanger, Robichon, Migicovsky, Copp & Vincent, 1963) seem to explain the established experimental data adequately. Talmage has postulated a dual action for PTH on bone:

(i) the increased transfer of calcium from bone to blood. This mechanism would be a result of increased osteolysis around osteocytes (Belanger *et al.*, 1963); and

(ii) upon more sustained application, the stimulation of osteoclast formation and thereby the rate of bone remodelling. The phenomenon of bone remodelling is most clearly seen in growing bones in areas adjacent to joints. Bone is resorbed and redeposited in different areas to maintain the contour of the bone shafts.

Further evidence has accumulated since this hypothesis was advanced. Increased RNA synthesis in bone cells has been observed after PTH administration *in vivo* (Park & Talmage, 1967; Owen & Bingham, 1968). The RNA may be the initiator of the synthesis of lysosomal proteins which would specifically produce osteolysis (Vaes, 1968). This osteolytic concept is substantiated by the observation of increased urinary excretion of the collagen breakdown product hydroxyproline in PTH-treated rats (c.f. Rasmussen & Feinblatt, 1971). Interestingly, PTH

has been shown to rapidly activate adenyl cyclase and increase production of cyclic adenosine 3', 5'-monophosphate (cyclic AMP) in suspensions of bone cells (Chase & Aurbach, 1968; Chase, Fedak & Aurbach, 1969). Wells & Lloyd (1968) drew these observations together into an attractive hypothesis. PTH was considered to stimulate the production of cyclic AMP which acted as a "second messenger", promoting RNA synthesis and the release and synthesis of lysosomal enzymes. These enzymes then produced osteolysis and calcium liberation. Since actinomycin-D will block the synthesis, but probably not the release, of the lysosomal enzymes, this mechanism could explain the biphasic response to PTH which was noted above (Rasmussen et al., 1964). The mechanism was further extended to include the mode of action of CT. This was postulated to reduce intracellular cyclic AMP, possibly by the activation of phosphodiesterase (see Section 5.4). This mechanism would accommodate the observation that actinomycin-D does not influence the action of CT upon bone (Tashjian, 1965). CT is discussed in detail in Section 4.4.

PTH has the dual actions on rat kidney of increasing tubular reabsorption of calcium and promoting phosphate excretion (Talmage & Kraintz, 1954). The former effect is probably obscured in vivo by the hypercalcaemia produced by PTH administration (Copp, 1969a). The phosphaturic action of PTH is exerted upon the distal tubule in the dog (Nicholson, 1959) and is not dependent upon the presence of vitamin D in rats (Rasmussen, DeLuca, Arnaud, Hawker & Von Stedingk, 1963; Arnaud, Rasmussen & Anast, 1966). Whether this phosphaturic effect is due to inhibition of tubular reabsorption of phosphate or to active secretion of phosphate by the distal tubules is not certain, but little convincing evidence for the latter has been advanced (Eisenberg, 1968).

The phosphaturia produced by physiological doses of PTH was preceded by a sharp increase in the renal excretion of cyclic AMP (Chase & Aurbach, 1967) suggesting that the cyclic nucleotide has a role in this action of PTH, in addition to that on the bone cells.

Absorption of calcium from the gut is generally believed to be enhanced by PTH, but this issue is often contentious (c.f. Talmage, 1967b). For example, although a very clear role for the hormone has been demonstrated by some workers in the rat (Rasmussen, 1959), dog (Cramer, 1963) and sheep (Care & Keynes, 1964), others have claimed that no effect is observable in rats (Wasserman & Comar, 1961).

The extraosseous effects of PTH, while interesting in themselves, must be considered only as adjuncts to the primary mechanism of calcium regulation, which is the balance between bone and fluid calcium. This of course does not denigrate the importance of calcium absorption and excretion.

The regulation of PTH secretion by a negative feedback of elevated blood calcium, proposed by McLean (1957), has now been confirmed in several species but never as elegantly as in the study by Potts, Buckle, Sherwood, Ramberg, Mayer, Kronfeld, Deftos, Care & Aurbach (1968). An inverse relationship was established between plasma calcium concentration and circulating PTH (measured by radioimmunoassay) with a precise line of best fit. The principle has been confirmed in man (Berson & Yallow, 1966) and pig (Arnaud, Littledike & Tsao, 1970). Furthermore, direct perfusion of the parathyroid glands in sheep and goats (Care, Sherwood, Potts & Aurbach, 1966) demonstrated that elevated calcium concentrations in the perfusate inhibited PTH secretion. It should be noted that in some conditions of extreme calcium perturbation non-proportional responses in circulating PTH have been observed (Sherwood, Mayer, Ramberg, Kronfeld, Aurbach & Potts, 1968).

Several studies using different in vitro systems have elaborated a number of mechanistic aspects of the calcium/parathyroid cell interaction:

(i) Rat parathyroids cultured in low calcium media for 24h exhibited morphological changes characteristic of increased metabolic activity. Protein synthesis and release was also increased. The protein secretion was similar to PTH in nature. High calcium media inhibited all these changes. It was concluded (Raisz, 1963; Raisz, Au & Stern, 1965) that the calcium ion regulated parathyroid gland activity by actions at the cell surface;

(ii) The action of calcium ions upon bovine parathyroid explants was shown to be mediated by cyclic AMP (Sherwood, Lundberg, Targovnik, Rodman & Seyfer, 1971); and

(iii) Superfusion of porcine parathyroid slices with a medium low in calcium or magnesium, or containing CT, increased PTH secretion. Calcium and magnesium-rich superfusate inhibited secretion. Ultrastructural changes were observed to parallel the changes in PTH secretion rate. It was found possible to demonstrate two phases in the secretory response to low calcium concentrations. The initial phase was independent of protein synthesis while the succeeding phase was not. Other observations indicated the distinct identities of these two phases, which were postulated to be due to the release of stored PTH and the subsequent release of newly-synthesized hormone (Oldham, Fischer, Capen, Sizemore & Arnaud, 1971).

Rat PTH is at present not quantifiable by radioimmunoassay and thus relationships between plasma calcium concentration and PTH release have only been established by the use of less precise bioassay measurements of the hormone (c.f. Raisz, 1963). However, the in vitro observations of Raisz (Raisz, 1963; Raisz et al., 1965), described above, and a large body of evidence in the in vivo situation strongly suggest that

the general principles of the regulation of PTH secretion which have been previously outlined also apply to the rat (c.f. Talmage, 1967b; Copp, 1969a).

In summary, PTH has been shown to regulate the concentration of ionised calcium in body fluids in conjunction with CT (Section 4.4) and vitamin D (Section 4.5). The major function of PTH is to oppose the trend which leads to calcium deposition in bone mineral by increasing bone resorption. The hormone is also thought to regulate bone remodelling and is known to have subsidiary actions upon gut and kidney.

4.4 Calcitonin (CT).

The relationship between calcium level in the body fluids and PTH secretion would appear to be sufficient to explain the long term control of hypercalcaemic episodes (c.f. McLean, 1957). However, experimental observation by Copp, Davidson & Cheney (1961) suggested the existence of a hormone which could control hypercalcaemia in the short-term, i.e. a naturally-occurring hypocalcaemic agent. Copp termed this hormone calcitonin and believed that it was of parathyroid origin.

Further studies soon demonstrated that the major source of calcitonin was the thyroid gland:

(i) parathyroid gland removal by cautery produced a more rapid hypocalcaemia in rats than did surgical excision (Hirsch, Gauthier & Munson, 1963);

(ii) thyroid extracts produced marked hypocalcaemia when injected into rats (Hirsch et al., 1963, 1964);

(iii) blood calcium level after calcium infusion rose to much higher levels in rats which had been surgically thyroidectomised with reimplantation of the parathyroids (TX) than in PTH animals (Talmage, Neuenschwander & Kraintz, 1965); and

(iv) finally, and probably most unequivocally, the importance of the

thyroid in the control of hypercalcaemia was demonstrated by Care (1965). Hypercalcaemic perfusion of the pig thyroid, which was shown to contain no parathyroid tissue, produced a profound hypocalcaemic response.

It should be noted that CT has been extracted from the parathyroid tissue of dogs and rabbits (Copp & Parkes, 1968), but many studies have established that the hormone is chiefly produced and stored in cells in the thyroid gland. These cells are distinct from the thyroxine-synthesizing follicular cells and have been designated "C" cells (Pearse, 1966). Immunofluorescent techniques have revealed the presence of CT in dog and pig C cells (Bussolati & Pearse, 1967). These cells underwent degranulation during hypercalcaemia (Pearse, 1966). Embryologic and phylogenetic evidence (see Copp & Parkes, 1968, Copp, 1969a for reviews) suggests that the C cells are closely related to ultimobranchial cells and the term calcitonin is therefore preferable to "thyrocalcitonin" (suggested by Hirsch et al., 1964).

Both bioassays and radioimmunoassays for CT have been utilised to investigate the control of CT secretion. Linear relationships between plasma calcium concentration at and above normal levels and CT secretion rate have been demonstrated in pigs (Care, Cooper, Duncan & Orimo, 1968; Cooper, Deftos & Potts, 1971; West, O'Riordan, Copp, Bates & Care, 1973), sheep (Care et al., 1968), rabbits (Lee, Deftos & Potts, 1969) and calves (Care, Bates, Philippo, Lequin, Hackeng, Barlet & Larvor, 1970). CT is secreted continuously at normal plasma calcium concentrations in these species and in rats (Klein & Talmage, 1968).

The primary target organ for CT is the skeleton (Munson, 1971) although some subsidiary actions upon kidney have been reported (Robinson, Martin & MacIntyre, 1966). The hormone is not thought to significantly influence intestinal calcium absorption (Cramer, Parkes & Copp, 1969). Numerous studies in a variety of species, both in vivo

and _in vitro_, have demonstrated that CT has an inhibitory effect upon PTH-induced bone resorption (Aliapoulios, Goldhaber & Munson, 1966; Martin _et al._, 1966; Milhaud & Moukhtar, 1966; Munson, 1971). However, since the hypocalcaemic action of CT is still manifested in aparathyroid rats (c.f. Munson, 1971) it is clear that the hormone also exhibits other properties. The work of Foster and his colleagues (Foster, Doyle, Bordier & Matrajt, 1966) and Pechet _et al._ (Pechet, Bobadilla, Carroll & Hesse, 1967) _in vivo_, and Gaillard (1967) _in vitro_, strongly suggested that CT actively promoted bone accretion.

CT has been reported to increase calcium excretion in the urine of rats (Aldred, Kleszynski & Bastian, 1970) and to be phosphaturic in PTX rats (Martin _et al._, 1966). The significance of these observations has not been clearly established.

It is tempting to postulate that the actions of CT upon its target cells are connected with its ability to "pump" calcium out of cells (Borle, 1967, 1968; Rasmussen & Tenenhouse, 1970; Copp, 1973; Whitfield, Rixon, MacManus & Balk, 1973c) but this has not been established. It does, however, seem that activation of 'C' cell adenyl cyclase is a prerequisite for the stimulatory effects upon CT secretion of high calcium concentrations and a number of hormones, _in vitro_ at least (Care, Bates & Gitelman, 1970; Care, Bruce, Boelkins, Kenny, Conaway & Anast, 1971).

Although CT has been clearly shown to have an important role in the reduction of the hypercalcaemia elicited by administration of calcium salts, PTH and vitamin D (c.f. Munson, 1971), its true physiological role is less well understood. As discussed previously (Section 4.3), it seems certain that the animal constantly needs to counter the trend towards hypocalcaemia and that naturally-occurring hypercalcaemic episodes are likely to be infrequent. Furthermore, the hypocalcaemic

response to CT in adult rats is only some 5% of that in 30 day old animals (Copp, 1969b), presumably because bone resorption is much reduced with age (Copp, 1969a). Thyroidectomy (with thyroxine replacement therapy) has failed to produce gross perturbations in calcium homeostasis in adults of several species (c.f. Care, 1969; Copp, 1969a). Thus it can be concluded that CT has, at best, only a minor role in calcium homeostasis compared to PTH (and vitamin D). However, there are three situations in which CT is likely to be of importance:

(i) Hypercalcaemia resulting from parathyroid hyperactivity;

(ii) Excessive skeletal resorption in females during pregnancy and lactation; and

(iii) Short-term hypercalcaemias in animals which ingest food after a period of starvation. Such an animal is the rat, which exhibits a distinct circadian periodicity in food intake (Besch, 1970).

Prolonged hypercalcaemia might result in renal and soft-tissue calcification with, possibly, reduced neuromuscular excitability. CT status in hyperparathyroid syndromes has not been satisfactorily investigated. TX rats maintained on low calcium diets have been shown (Lewis et al., 1971) to have lower skeletal calcium after pregnancy and lactation than intact animals maintained on the same regime. Furthermore, in rats (Gray & Munson, 1969; Milhaud, Perault-Staub & Staub, 1972), sheep (Phillippo et al., 1972) and pigs (Swaminathan et al., 1973) CT has been shown to play a significant role in the control of post-prandial hypercalcaemia. The hormone is probably of most significance in young animals and in fishes which exist in high concentrations of ionised calcium in sea water. It is therefore of considerable phylogenetic interest.

In summary, CT is a hypocalcaemic, hypophosphataemic agent of

thyroid origin. Its primary action is upon the skeleton where it inhibits bone resorption and promotes bone accretion. It is continually secreted at physiological blood calcium levels and this, and other evidence, suggests that it has significance in calcium homeostasis in mammals. Its rate of secretion in several species is directly proportional to blood calcium concentrations at and above normal levels.

4.5 Vitamin D.

The vital role played by vitamin D in calcium homeostasis was demonstrated by the observation that there was a requirement for the vitamin for the expression of the physiological action of PTH (Harrison *et al.*, 1958; Harrison & Harrison, 1961; Rasmussen *et al.*, 1963). Rats maintained on a diet containing no vitamin D were shown to become severely hypocalcaemic. This was reversible by administration of the vitamin.

The relationship between lack of exposure to sunlight, concomitant with inadequate diet, and the development of the "soft-bone" diseases "Ricketts" and osteomalcia has long been established. These diseases are characterised by the failure of bone calcification to keep pace with the synthesis and deposition of new, organic bone matrix (DeLuca, 1967). Thus it might be inferred that vitamin D acted at the calcification sites. However, it now seems certain that the decrease in bone calcification is secondary to the insufficiencies of calcium and phosphate homeostasis in conditions of vitamin D deficiency (DeLuca, 1967, 1969, 1971). The vitamin appears to be essential to prevent the nett movement of calcium from the plasma into bone (see Section 4.1). Its action appears to be stimulatory upon both intestinal calcium absorption (Wasserman & Kalfelz, 1962) and bone mineral mobilisation (DeLuca, 1967).

Two sources of the hormone appear to be of importance, namely dietary and endogenously synthesized vitamin. The vitamin is produced in the skin by ultraviolet irradiation of the pro-vitamin 7-dehydrocholesterol. The details of the synthesis have not been studied *in vivo*, but *in vitro* the pro-vitamin is converted to "pre-vitamin D" which is then slowly converted to vitamin D_3 (cholecalciferol) without the necessity for further ultraviolet exposure (Avioli & Haddad, 1973). The amounts of the vitamin thus produced are considered to be significant in man (Avioli & Haddad, 1973) and DeLuca (1971) has suggested that the dietary requirement for vitamin D_3 is an artefact of civilization. This can hardly apply to most animals, in which skin coverage by hair is probably equivalent to man's clothing. Furthermore, gross evidence of vitamin D deficiency is readily obtained in animals maintained on a vitamin D-free diet (Harrison *et al.*, 1958; Rasmussen *et al.*, 1963). The status of endogenously synthesized vitamin D_3 is not fully discussed by these workers. It therefore seems that although the metabolites of vitamin D_3 exhibit the characteristics of hormones, the parent molecule does not do so.

The lag between the administration of vitamin D_3 and the appearance of enhanced intestinal calcium absorption suggested either that the transport time to the target site might be prolonged or that conversion might take place to yield a metabolically active derivative (DeLuca, 1967). The former possibility was subsequently eliminated (Neville & DeLuca, 1966) and a number of investigations have confirmed the validity of the latter hypothesis. Vitamin D_3 is metabolised in the liver (Horsting & DeLuca, 1969; Ponchon & DeLuca, 1969; Ponchon, Kennan & DeLuca, 1969), probably by an oxygen-dependent mitochondrial enzyme system (Horsting & DeLuca, 1969), to 25-hydroxycholecalciferol (25-HCC). This hydroxylation is subject to marked product inhibition

(Horsting & DeLuca, 1969) and this probably constitutes an important physiological regulation system (DeLuca, 1971). The hydroxylated configuration is the major circulating form of vitamin D_3 and is capable of stimulating both intestinal calcium absorption (Olson & DeLuca, 1969) and bone mineral mobilisation (Raisz, Trummel & Simmons, 1972).

Further hydroxylation of 25-HCC by a kidney mitochondrial system to either 1, 25-dihydrocholecalciferol (1, 25-DHCC) or 24, 25-dihydrocholecalciferol (24, 25-DHCC) has been established (Gray, Boyle & DeLuca, 1971). Evidence has accumulated which suggests that this process may be subject to regulation by circulating calcium, PTH and CT (Garabedian, Holick, DeLuca & Boyle, 1972; Rasmussen, Wong, Bible & Goodman, 1972). For example, TPTX animals do not synthesize 1,25-DHCC, although this process is restored by administration of PTH (Garabedian et al., 1972). These observations have been schematised (c.f. Avioli & Haddad, 1973). Hypocalcaemia was considered to stimulate release of PTH which stimulated the conversion of 25-HCC to 1,25-DHCC. The dihydroxyl metabolite then enhanced intestinal absorption and bone mobilisation of calcium. Hypercalcaemia was postulated to have an opposite effect, possibly involving CT and the preferential synthesis of 24,25-DHCC, which has a very low activity. This hypothesis has been supported by some experimental observations (Garabedian et al., 1972; Rasmussen et al., 1972) but strongly refuted by others (Galante, Colston, MacAuley & MacIntyre, 1972). The latter workers have demonstrated a suppression by PTH of the hydroxylation of 25-HCC to 1,25-DHCC, with a concomitant preferential synthesis of 24,25-DHCC. Thus the hypothesis outlined above awaits further clarification.

The 1,25-dihydroxylated form of cholecalciferol is some 100 times more potent than 25-HCC for the production of bone calcium mobilisation in vitro (Raisz, Trummel, Holick & DeLuca, 1972) and approximately

twice as effective in stimulating intestinal calcium transport (Myrtle & Norman, 1971). It seems likely that 1,25-DHCC is the "effector" metabolite of vitamin D_3 (Avioli & Haddad, 1973) but its mechanism of action on bone and intestine has not been well established. A number of schemes involving carrier systems, either enzymatic or simply proteinacious in nature, have been suggested to explain the action of vitamin D_3 metabolites on intestinal calcium transport (Taylor & Wasserman, 1967; DeLuca, 1969, 1971). Other studies (Patrick, 1973) have suggested that 1,25-DHCC increases the entry of calcium to the brush-border epithelium and others are consistent with a trophic effect upon intestinal mucosa (Urban & Schedl, 1969; Spielvogel, Farley & Norman, 1972) which could increase the absorptive area. A combination of these mechanisms may be most likely. The action of metabolites upon calcium resorption from bone has been visualized by DeLuca (1971), but this explanation does not encompass some of the data presented by Talmage (1969).

Arguments about the relative importances of cholecalciferol and PTH in calcium homeostasis are not particularly fruitful and thus it is preferable simply to observe that the presence of both is required for the maintenance of bone formation and the regulation of calcium in body fluids.

In summary, vitamin D_3 (cholecalciferol) is probably derived from both dietary and biogenic sources. The vitamin undergoes hydroxylation to 25-hydroxycholecalciferol (25-HCC) in liver and this derivative is then further metabolised in the kidney. The most active metabolite (on intestinal calcium absorption and bone resorption) is 1,25-dihydroxy-cholecalciferol (1, 25-DHCC). PTH, CT and 1,25-DHCC are undoubtedly functionally integrated and interdependent.

4.6 Summary.

The actions of the calcium homeostatic hormones which have been discussed above are directed towards the maintenance of the concentration of the calcium ion in body fluids within physiologically desirable limits and to the control of bone formation and mechanical strength. Evidence discussed previously (Section 4.1) demonstrates that via their control of calcium homeostasis these hormones regulate, or at least maintain, a wide range of vital body processes. In addition, evidence to be presented (Section 5) will further emphasize their role in the control of cell proliferation in a number of tissues.

PTH opposes the tendency of calcium to become concentrated in the skeleton from the plasma by stimulating bone resorption. The hormone also stimulates intestinal absorption and renal reabsorption of calcium and is phosphaturic. Bone remodelling is also strongly influenced by PTH. Evidence has been presented which suggests a physiological role for CT in bone deposition and the protection of skeletal mineral. Vitamin D is, as yet, less completely investigated than PTH or CT. It is, however, established that the presence of vitamin D_3 or its metabolites is essential for the maintenance of calcium homeostasis. Manipulation of fluid calcium levels by surgery or injection of the above hormones was essential for the in vivo investigations described later in this study.

Section 5: Calcium, Hormones and the Control of Cell Proliferation.

5.1 Early observations.

A number of experimental observations indicated that some invertebrate and mammalian cell types were susceptible to the mitogenic action of the calcium ion (Hollingsworth, 1941; Heilbrun, 1952; St. Amand, Anderson & Gaulden, 1960; Whitfield & Rixon, 1962). Despite the observations that administration of parathyroid extract (Rixon & Whitfield, 1961) or calcium (Rixon & Whitfield, 1963) to irradiated rats increased their survival rate, the significance of these early observations was not full expounded until the demonstration that injections of calcium chloride heightened mitotic activity in the rat thymus gland (Perris & Whitfield, 1967a). Subsequent studies leave little doubt that the calcium homeostatic system constitutes a crucial control mechanism for the proliferation in haemopoietic and lymphopoietic tissues of the rat <u>in vivo</u> (Perris, 1971; Whitfield, Rixon, MacManus & Balk, 1973c; also see Results, Chapters 1, 2, 3, 4 and 6).

5.2 General Effects of Hormones and Ions <u>In Vivo</u>

The initial demonstration that injection of calcium stimulated thymocyte proliferation (Perris & Whitfield, 1967a) was followed by other studies which extended the observation to the bone marrow (Perris, Whitfield & Rixon, 1967; Perris & Whitfield, 1967b). Concurrently, extracts of parathyroid hormone were shown to share these properties, probably via the elevation of circulating calcium levels (c.f. Section 4.3). A physiological role for the parathyroid gland in the maintenance of bone marrow and thymic mitotic activity and cellularity was established by a series of experiments with aparathyroid rats. Extirpation of the parathyroid gland, with subsequent maintenance of the animal on a low calcium diet (see General Methodology, Section 4.2),

was shown to:

(i) Reduce mitotic activity in thymus and bone marrow (Rixon, 1968; Perris, Weiss & Whitfield, 1970; Perris et al., 1971; Perris & Whitfield, 1971; Rixon & Whitfield, 1972a; see also Results, Chapters 1, 2 and 3);

(ii) Reduce cellularity in thymus and bone marrow (Perris et al., 1970; Rixon & Whitfield, 1972a); and

(iii) Reduce thymic weight (Perris et al., 1970).

Injection of calcium salts or PTH, or addition of calcium salts to drinking water, partially or wholly restored thymus and bone marrow mitotic activity in these rats for a transient period (Perris et al., 1970; Perris & Whitfield, 1971). Significantly, the atrophic and hypoplastic effects of parathyroidectomy (PTX) were not evident in animals which had been maintained on a diet containing the normal amount of calcium (Rixon & Whitfield, 1972a; Rixon, unpublished observations quoted by Whitfield et al., 1973c). The plasma calcium levels of these rats were at, or near, normal levels (c.f. General Methodology, Section 4.2). Thus the extracellular calcium ion concentration was obviously the determining factor in the phenomena described above. It seems likely from these observations that PTH in vivo has no direct role in the maintenance of bone marrow and thymus proliferation, but rather that its calcium homeostatic activity determines its demonstrably important regulation of mitotic activity in these two tissues. The mitotic activity of PTH in vitro is discussed later (Section 5.3).

Recent studies have indicated that parathyroid-dependent processes may also govern proliferation in regenerating hepatic tissue (Rixon & Whitfield, 1972b). The pattern of extracellular calcium movement after partial hepatectomy was distinctive. Directly after the operation a hypocalcaemic phase ensued which persisted for up to 24h. The mag-

nitude of the decline in plasma calcium conentrations (10%) was much greater than that observed in sham-operated animals (2-3%). At the termination of the hypocalcaemic phase DNA synthesis and cell proliferation were observed. The appearance of these two phenomena was severely delayed in aparathyroid animals and, in addition, their magnitudes were significantly reduced. A further tissue which may be influenced by the extracellular calcium ion environment is the rapidly proliferating epithelial gut mucosa (Mellon, 1974; Smith, 1974). The injection of calcium salts and PTH stimulated cell proliferation in jejunal and duodenal mucosa (Smith, 1974) and parathyroidectomy reduced proliferation in these tissues (Mellon, 1974).

Thus in three renewing tissues (bone marrow, thymus and intestinal mucosa) and one regenerating tissue (liver) the calcium homeostatic system has been shown to profoundly influence the rate of cell proliferation. The studies of liver regeneration and intestinal mucosal proliferation are as yet only in the preliminary stage. Further studies upon the physiological significance of the observations discussed above are considered in greater depth later (Sections 5.5 and 5.6; see also Results, Chapters 1, 2, 3, 4 and 6).

5.3 General Effects of Hormones and Ions *In Vitro*.

Suspensions of rat thymic lymphocytes maintained *in vitro* have been extensively used for the elucidation of the mechanism of action of various mitotic stimulants. In particular, mechanisms have been described which attempt to explain the mitogenic or anti-mitogenic actions of the calcium ion, PTH and CT (Whitfield *et al.*, 1973c). In addition, the influences of a wide range of hormones, ions, cyclic nucleotides, detergents and biogenic amines have been investigated (Whitfield & Youdale, 1966; Whitfield, Perris & Youdale, 1968; MacManus

& Whitfield, 1969a, b; Whitfield et al., 1969a, c; Whitfield, Rixon, Perris & Youdale, 1969b; Whitfield et al., 1970a, b; MacManus et al. 1971a; MacManus, Whitfield & Braceland, 1971b; Whitfield & MacManus, 1972; Whitfield, MacManus, Braceland & Gillan, 1972a, b; Whitfield, MacManus, Franks, Braceland & Gillan, 1972c; Whitfield, MacManus & Gillan, 1973a, b). These studies have been performed using rat thymic lymphocyte populations for predominantly practical reasons, i.e. the high rate of proliferation in thymic lymphoblasts (see Section 3.3) and the ease of thymocyte culture in simple media (General Methodology, Section 7.2). Thus the thymic lymphoblast has been used as a test vehicle for the elucidation of mitogenic activities and this has often been at the expense of considerations of the significance of these observations to the whole animal. For example, the extracellular concentrations of some hormones and cyclic nucleotides which were shown to stimulate thymic lymphoblast proliferation (MacManus & Whitfield, 1969a; Whitfield et al., 1969a, 1970a) were unphysiological. The effects of increased calcium ion concentrations were, however, exerted in physiological ranges (Whitfield et al., 1969b; MacManus & Whitfield, 1971) which is a further indication that this ion is of importance in the control of thymic lymphoblast proliferation *in vivo*.

Mitotic activity and in some cases DNA synthesis in rat thymocyte populations maintained *in vitro* has been shown to be stimulated by elevated concentrations of magnesium ions (Morgan & Perris, 1974), by PTH (Whitfield et al., 1969a, 1970b; Whitfield, MacManus, Youdale & Franks, 1971), growth hormone (MacManus & Whitfield, 1969b; Whitfield et al., 1969a, c), vasopressin (Whitfield et al., 1969a, 1970a), oxytocin (Whitfield et al., 1969a), prolactin (Whitfield et al., 1969a), adrenaline (MacManus et al., 1971a), prostaglandin E_1 (Franks, MacManus & Whitfield, 1971; Whitfield et al., 1972a, b, c) and exogenous cyclic AMP (MacManus & Whitfield, 1969a). The effects of these and other

mitogenic agents both in vitro and in vivo, are summarised in Table 1. Many of the above observations are probably of little or no significance in the in vivo situation because of the non-physiological doses used. Indeed, an additional criticism which may be levelled at all in vitro culture systems is that they may poorly reflect the behaviour of the cells in situ. However, the stimulation of thymocyte proliferation by elevated concentrations of the calcium ion has been established in vivo (Section 5.2) as well as in the in vitro studies reported above. Thus there is circumstantial evidence that in this respect at least the behaviour of the cultured thymocyte simulates the activity of thymocytes in the whole animal. These considerations do not of course apply to studies in which the suspended thymocytes are used merely as a vehicle for the dissection of various mitogenic mechanisms, provided that the conclusions arrived at are not extrapolated directly to the in vivo situation without further investigations. An analogy might be drawn between the use of thymocyte cultures in this way and the use of HeLa cell cultures for investigations of cellular phenomena.

The studies which have been reported above have led to the development of a number of hypotheses to explain the mechanisms of action of a wide range of mitogenic agents (Whitfield et al., 1973c). The basic theories, which are discussed in Section 5.4 below, appear to have relevance for the in vivo situation. This argument will be developed later (Sections 5.4, 5.5 and 5.6).

The mitogenic properties of the calcium ion have also been demonstrated in vitro in rat bone marrow cells (Morton, 1968; Morton, Perris & Whitfield, 1968; see also Results, Chapter 5). Nucleated cell numbers, reticulocyte numbers and mitotic activity in bone marrow cultures were all increased by elevated extracellular calcium ion concentrations. These observations parallel those described in Section 5.2 above and others (Whitfield & Perris, 1971) recorded in vivo in

Table 1: Calcium and Cell Proliferation in Tissues of the Rat.

(a) In Vivo.

Observation	References
A. Elevation of extracellular Ca^{++} concentration by injection of $CaCl_2$	
1. Stimulates thymocyte proliferation.	Perris & Whitfield, 1967a. Perris et al. 1967, 1968. Smith et al. 1974
2. Stimulates bone marrow cellular proliferation.	Perris et al. 1967 Rixon, 1968 Perris & Whitfield, 1971. Smith et al. 1974.
3. Stimulates reticulocyte formation in bone marrow.	Perris & Whitfield, 1971.
4. Stimulates ^{59}Fe incorporation into red blood cells.	Perris & Whitfield, 1971.
5. Stimulates cellular proliferation in intestinal mucosum.	Smith, unpublished.
6. Enhances antibody response.	Braun et al. 1970.
7. Increases survival of irradiated rats.	Rixon & Whitfield, 1963.
B. Elevation of extracellular Ca^{++} concentration by injection of PTH.	
1. Stimulates thymocyte proliferation	Perris et al. 1967, 1970.
2. Stimulates bone marrow cellular proliferation.	Perris et al. 1967, 1971. Perris & Whitfield, 1971.
3. Stimulates reticulocyte formation in bone marrow.	Perris & Whitfield, 1971.

Table 1: continued....

	Observation	References
4.	Stimulates ^{59}Fe incorporation into red blood cells.	Perris & Whitfield, 1971.
5.	Stimulates cellular proliferation in intestinal mucosa .	Smith, unpublished.
6.	Increases survival of irradiated rats.	Rixon & Whitfield, 1961.
C.	<u>Reduction of extracellular Ca^{++} concentration by parathyroidectomy</u>.	
1.	Reduces thymocyte proliferation.	Perris *et al.* 1970. Hunt & Perris, 1974.
2.	Produces thymic atrophy.	Perris *et al.* 1970.
3.	Reduces bone marrow cellular proliferation.	Rixon, 1968. Perris & Whitfield, 1971. Perris *et al.* 1971. Rixon & Whitfield, 1972a. Hunt & Perris, 1973, 1974.
4.	Produces marrow hypoplasia.	Rixon & Whitfield, 1972a.
5.	Reduces reticulocyte formation in bone marrow.	Perris & Whitfield, 1971.
6.	Reduces ^{59}Fe incorporation into red blood cells.	Perris & Whitfield, 1971.
7.	Abolishes mitogenic action of EPO in bone marrow.	Hunt & Perris, 1974.
8.	Abolishes circadian changes in bone marrow and thymus cellular proliferation.	Hunt & Perris, 1974.

Table 1: continued....

Observation	References
9. Increases time for post-haemorrhagic restoration of haematocrit.	Perris et al. 1971.
10. Abolishes increase in bone marrow mitosis 24h after haemorrhage.	Perris et al. 1971.
11. Reduces cellular proliferation in intestinal mucosa.	Mellon, unpublished.
12. Reduces and delays DNA synthesis and cellular proliferation in regenerating liver.	Rixon & Whitfield, 1972b.

(b) In Vitro.

Observation	References
A. Elevated extracellular calcium concentration.	
1. Stimulates thymocyte proliferation.	Whitfield et al. 1968, 1969a,b. Smith et al. 1974. Morgan & Perris, 1974.
2. Stimulates DNA synthesis in thymocytes.	Whitfield et al. 1971.
3. Stimulates proliferation of bone marrow cells.	Morton, 1968. Morton et al. 1968.
4. Stimulates reticulocyte production in bone marrow cultures.	Morton, 1968.
5. Stimulates proliferation of strain L mouse cells.	Yang & Morton, 1971.
6. Stimulates proliferation of chicken fibroblasts.	Balk, 1971.

Table 1: continued....

	Observations	References
B.	Agents which require the presence of extracellular calcium for expression of their mitogenic effects.	
1.	PTH	Whitfield et al. 1969a, 1971.
2.	Detergents, agmatine, poly-L-lysine	Whitfield et al. 1968.
3.	Growth hormone, neurohormones, prolactin.	Whitfield et al. 1969a,c.
4.	Cortisol.	Whitfield et al. 1973b.
5.	Acetylcholine, histamine, insulin.	Morgan, unpublished.

Figure 4, overleaf.

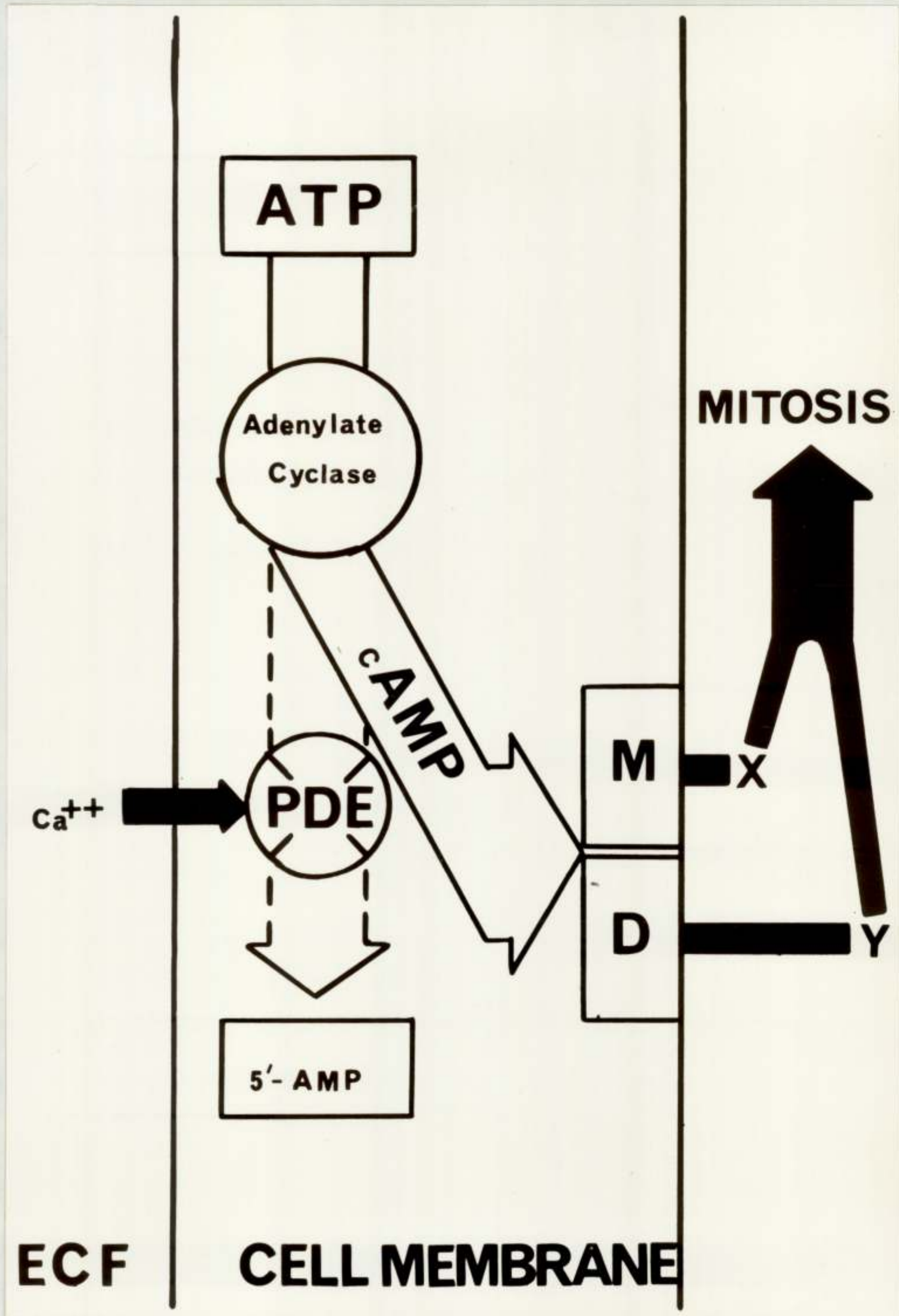


Figure 4: Proposed mechanism for the mitogenic action of calcium in the extracellular fluid (ECF) of thymic lymphoblasts (modified from Whitfield et al., 1973c). Exposure of the cell to Ca^{++} concentrations greater than 1.0mM inhibits the action of the membrane-bound phosphodiesterase (PDE) of cyclic adenosine 3', 5'-monophosphate (cAMP), which degrades cAMP to 5'-adenosine monophosphate (5'-AMP). Thus intramembranal concentrations of cAMP, which is formed from ATP by the action of adenylate cyclase, are elevated. The elevated cAMP level stimulates the mitogenic (M) and DNA-synthetic (D) components of a membrane activation site to produce initiators (X and Y) which enter the cell and trigger the mitogenic and DNA-synthetic processes.

bone marrow. No studies have been performed upon the possible mechanisms of action of the calcium ion in this in vitro bone marrow system (but see Results, Chapter 5).

5.4 Possible Mechanisms for the Mitogenic Activities of Calcium and for the Control of Cell Proliferation.

Whitfield and his associates (Whitfield et al., 1973c) have proposed that a complex skein of events occupies the time between the elevation of extracellular calcium ion concentration and the entry of thymic lymphoblasts into mitosis (Figure 4). The ubiquitous cyclic nucleotide adenosine 3', 5'-monophosphate (cyclic AMP) is deeply involved in these processes. This has been demonstrated by the use of a number of "tools" which interfere with cyclic AMP metabolism. Formation of cyclic AMP from adenosine 5'-triphosphate (ATP) and degradation to adenosine 5'-monophosphate (5'-AMP) are catalysed by the enzymes adenyl cyclase and phosphodiesterase (PDE) respectively (Robi son et al., 1971). Thus cyclic AMP levels in tissues may be influenced by agents which potentiate or inhibit either of these two enzymes. A number of hormones, e.g. glucagon or adrenocorticotrophic hormone (ACTH), are known to be adenylate cyclase stimulators (Robi son et al., 1971). Imidazole is known to stimulate PDE activity and theophylline and caffeine are known to be inhibitory (Robi son et al., 1971). Thus administration of imidazole to tissues lowers cyclic AMP levels and administration of caffeine has the reverse effect.

If mitotic activity in thymic lymphoblasts in vivo was solely dependent upon the extracellular calcium ion environment it would be difficult to understand how DNA synthesis might be initiated in the absence of appreciable changes in calcium homeostasis. Elevated calcium ion concentrations have been demonstrated to initiate DNA synthesis in thymic lymphoblasts in vitro, 3-4h prior to increased mitotic activity (Whitfield et al., 1969b; MacManus & Whitfield,

1971; Whitfield et al., 1971). However, since mitosis proceeds in vitro in the complete absence of extracellular calcium (Whitfield et al., 1971) and in vivo in aparathyroid rats in which plasma ionised calcium levels are reduced by 30-50% (Perris et al., 1970; see also Results, Chapter 1) it is clear that the maintenance of mitotic activity in thymic lymphoblasts is not solely dependent upon the extracellular calcium ion status. It is envisaged (Whitfield et al., 1973c) that, in conditions of balanced calcium homeostasis, endogenously generated increases in cell membrane permeability to the calcium ion may facilitate its entry to the cell. This would occur at some pertinent stage in the cell cycle (i.e. probably G_1) and result in the initiation of DNA synthesis and ultimately the mitotic event. The mitogenically permissive (for calcium) actions of a variety of membrane-active agents (Whitfield et al., 1968) may constitute support for this hypothesis.

The mitogenic action of calcium appears to be mediated via an increase in intracellular cyclic AMP level, for clear parallels have been observed between extracellular calcium ion concentrations (over the range 0 - 2.4mM, i.e. 0 - 9.6mg/100ml) and cellular cyclic AMP content, DNA synthetic rate and cell proliferation (MacManus & Whitfield, 1971; Whitfield et al., 1971). Furthermore, imidazole and caffeine (see above) inhibited and potentiated, respectively, calcium-induced mitogenesis (MacManus & Whitfield, 1971). In addition, exogenous cyclic AMP in low doses stimulated proliferation of thymocyte populations in vitro and bone marrow and thymus cells in vivo (Rixon et al., 1970; Whitfield et al., 1971). Mitogenic concentrations of calcium have been shown to inhibit both adenyl cyclase and PDE activities in broken cell preparations (MacManus & Whitfield, 1971;

Whitfield *et al.*, 1971). Although the formation of intracellular cyclic AMP is inhibited by the calcium ion, it seems likely that this effect is less significant than that which inhibits the destruction of the nucleotide; thus the nett result is an increase in intracellular cyclic AMP. The effects of imidazole and caffeine (see above) upon calcium-induced mitogenesis would also tend to support this hypothesis.

Elevated extracellular calcium ion concentrations, or changes in membrane permeability, are thought (Whitfield *et al.*, 1973c) to allow the ion access to a membrane bound PDE. Inhibition of the activity of this enzyme would then increase intramembrane cyclic AMP level. The elevated nucleotide concentration is then envisaged to cue a membrane activation site which initiates the DNA-synthetic and mitotic processes (Whitfield *et al.*, 1972a, b). This scheme is illustrated in Figure 4.

Further studies (Whitfield *et al.*, 1972a, b, c; Whitfield & MacManus, 1972) have demonstrated another action for calcium, in the generation of a mitotic response to a stimulant such as prostaglandin E_1. The mitogenic, but not the DNA-synthetic, process switched on by the proposed activation site appeared to be inhibited by high intracellular concentrations of calcium. It appeared possible that when intramembrane cyclic AMP levels were high under certain circumstances that some of the nucleotide may have diffused into the cytoplasm and released calcium from intracellular binding sites (e.g. mitochondria). If intracellular bound calcium was high, the amounts of the ion liberated may have been high enough to inhibit the development of the mitogenic event, though not that of the DNA-synthetic event (Whitfield *et al.*, 1972a, b). Thus in different combinations of circumstances cyclic AMP might have a stimulatory or an inhibitory role in proliferative events. These observations, and those which demonstrate the effects of guanosine

Figure 5 overleaf.

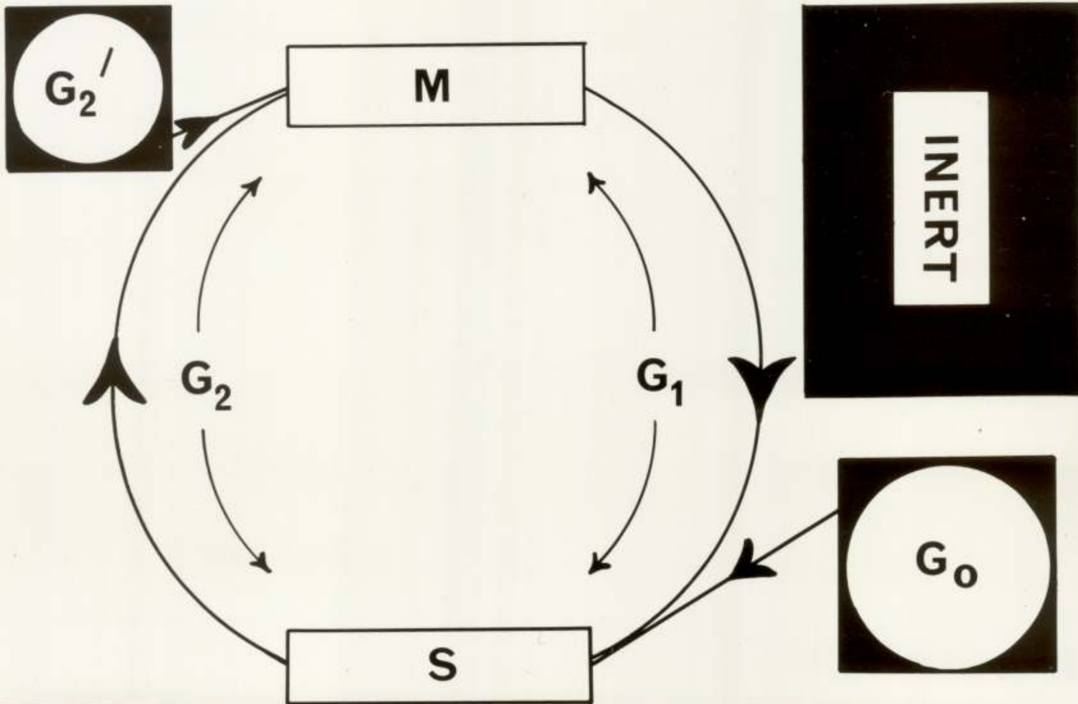


Figure 5: The cell cycle (after Howard & Pelc, 1953). Nomenclature discussed in text (General Methodology, Section 6.3). Scale of diagram does not indicate relative proportions of populations.

3', 5'-monophosphate and CT upon prostaglandin-stimulated thymocyte proliferation (Whitfield et al., 1972a; Whitfield & MacManus, 1972), make attractive the series of fine controls which can be visualised in terms of these mechanisms. However, once again it must be emphasized that these observations have still been insuficiently examined in their true physiological context.

The parallels between the effects of elevation of extracellular ionised calcium concentration upon thymic lymphoblast proliferation in vitro and in vivo (Perris & Whitfield, 1967a; Whitfield et al. 1969b; MacManus & Whitfield, 1971), and between the mitogenic effects of low doses of exogenous cyclic AMP in culture and in the whole animal (Rixon et al., 1970; Whitfield et al., 1971), suggest that the basic mechanisms elaborated by Whitfield and his colleagues are of significance in the control of mitotic activity in vivo. The hypothesis explains how both normal and supranormal thymic mitosis might be controlled by the calcium homeostatic system, via the extracellular calcium ion concentration. Stimulation of thymocyte proliferation might occur by actions on cycling cells in G_1 and also by recruitment of cells from the G_0 phase into the cycle at the G_1/S interface.

Observations on the possible mechanisms of calcium action on bone marrow cells have been limited (Rixon et al., 1970). This problem has been considered in the present study (Results, Chapter 5).

5.5 The Physiological Significance of Interrelationships Between the Calcium Homeostatic System and the Thymus.

The present poor understanding of the physiological importance of the thymus gland militates against definition of a role for the calcium homeostatic system in the immune response. However, a number of scattered observations have been made which provide a basis for the further

studies which are being pursued by other workers (Mekori & Perris, 1974).

Phytohaemagglutinin (PHA) transforms small, mitotically inert circulating lymphocytes into lymphoblasts, which are capable of DNA synthesis and cell proliferation. When calcium was absent from the medium, PHA treatment did not initiate DNA synthesis, whereas when the ion was present PHA treatment produced a sharp increment in uptake of calcium into the lymphocyte (Ashershon, Davey & Goodford, 1970; Whitney & Sutherland, 1972). Increases in intracellular cyclic AMP formation were observed after PHA treatment of human lymphocytes (Smith, Steiner, Newberry & Parker, 1971). These data have been interpreted by Whitfield et al., 1973c as evidence that calcium may promote transformation of (non-cycling) stem cells of bone marrow origin within the thymus to yield thymocyte progenitor cells. It is certain that the calcium homeostatic system crucially influences the subsequent proliferation of these progenitor cells (see Section 3.3).

Interesting observations were made by Braun, Ishizuka & Seeman, (1970) on the influences of calcium and CT upon the development of humoral antibody synthesis (in mice) in response to sheep red blood cells (SRBCs). Injection of calcium conjointly with, or within 6h of, injection of SRBCs stimulated the numbers of antibody-forming cells found in the spleen 48h or 72h later. Injection of the hormone CT, which acts to reduce plasma calcium levels (see Section 4.4), had directly opposite effects. Injection of calcium before, or 12-24h after, SRBCs was demonstrated to reduce the numbers of antibody-forming cells in the spleen at 48h. This effect was reversed when the response was measured at 72h. The authors have explained the inhibitory actions of calcium and CT by references to membrane changes associated with antigen-macrophage-lymphocyte interactions. However, they were not able to explain the stimulatory effect of calcium injections upon antibody-producing cell numbers in the spleen. It is

tempting to speculate that a mitogenic influence of calcium upon antibody-forming cell precursors could explain these observations. Calcitonin could possibly inhibit mitogenesis in these precursors, either by decreasing extracellular ionised calcium concentration (Hirsch, Voelkel & Munson, 1964), or by a direct action (Whitfield et al., 1972c). These possibilities are under investigation at present (Mekori & Perris, 1974).

5.6 The Physiological Significance of the Interrelationships Between the Calcium Homeostatic System and the Bone Marrow.

The observations discussed previously (Section 5.2) indicate that the calcium homeostatic system has a significant role in the control of cell proliferation in the bone marrow. However, the bone marrow contains dividing cells of at least four haemopoietic series (Section 2.10; Figure 3) and it is therefore necessary to distinguish whether the calcium ion has a non-specific effect upon all or a limited action upon particular lines.

The response to parathyroidectomy or thyroparathyroidectomy observed in the bone marrow is a significant decrease in mitotic activity within 7days(Rixon, 1968; Perris & Whitfield, 1971; Rixon & Whitfield, 1972a; see also Results, Chapters 1, 2 and 3). Furthermore, it has been shown that this mitotic depression was accompanied by a marked hypoplasia of the bone marrow (Rixon & Whitfield, 1972a). This reduction in cellularity was limited to the erythroid and lymphoid sub-populations. The myeloid series was not affected and the thromboid series (which was not investigated) is not characterised by extensive cell proliferation because a multiplication factor is inherent in the liberation of some 1000 platelets from each megakaryocyte (Lajtha, 1970; Section 2, 10; Figure 3). The parathyroid deprived animal also exhibited a severely depressed occurrence of reticulocytes in bone marrow and, subsequently,

peripheral blood, and significantly decreased incorporation of radioactive iron into peripheral red blood cells (Perris & Whitfield, 1971). Regular injections of $CaCl_2$ or PTH wholly or partially reversed these parathyroid-dependent trends and were also found to stimulate reticulocyte production and ^{59}Fe uptake in intact animals. These latter effects were also manifested in nephrectomised animals, which cannot produce significant quantities of EPO (Zanjani et al., 1967a, b), and in polycythemic rats which have only negligible concentrations of EPO in the plasma (Adamson & Finch, 1968). Thus they were not dependent upon the production of EPO or the activation of inert circulating hormone (Perris & Whitfield, 1971).

The primary action of the calcium ion would therefore appear to be on the erythroid, and to some extent lymphoid, sub-populations of the bone marrow. The lymphoid series generally exhibits little mitotic activity until acted upon by a thymus-originating factor, which is probably more readily available in response to certain antigenic stimuli (Miller & Mitchell, 1967; Gregory & Lajtha, 1968; Section 3.2). The importance of the calcium homeostatic system in the control of erythropoiesis was further emphasized by studies on the erythropoietic response to haemorrhage (Perris et al., 1971; Perris & Whitfield, 1971). After a initial hypocalcaemic episode, plasma calcium levels rose following blood loss and remained elevated until haematocrit was restored to normal levels (Perris et al., 1971). Bone marrow mitotic activity paralleled the hypercalcaemia. Parathyroidectomy abolished the increases in plasma calcium and bone marrow proliferation after haemorrhage and, although haematocrit was eventually re-established, the return to normal haematocrit values was considerably prolonged (Perris et al., 1971; Perris & Whitfield, 1971). The hypercalcaemic and proliferative phases in the intact animal were consequences of the loss of blood cells,

for they were also observed in animals which had been bled and then immediately reinfused with an equal volume of homologous plasma.

The systems concerned with calcium homeostasis have therefore been demonstrated to be intimately concerned with the two functional facets of erythropoiesis, namely the maintenance of constant circulating red cell mass and the ability to respond to hypoxic stimuli.

5.7 The Physiological Significance of the Interrelationships Between the Calcium Homeostatic System and Other Tissues.

Studies upon the influence of the calcium ion on proliferation in regenerating liver (Rixon & Whitfield, 1972b), intestinal mucosa (Mellon, 1974; Smith, 1974) and the immune system (Mekori & Perris, 1974) are largely in the preliminary stage. However, the inhibition by parathyroidectomy of DNA synthesis in regenerating liver and cell proliferation in this tissue and in intestinal mucosa (Mellon, 1974) suggests that calcium homeostasis may be related to these phenomena. Whitfield and his colleagues (Whitfield et al., 1973c) have speculated about the possible involvement of calcium in the cyclic AMP-dependent proliferation of kidney (Malamud & Malt, 1971; Taylor, 1971), parotid gland (Malamud, 1969) and adrenal cortical cells (Gill, 1972) in response to various stimuli. Since the dependence of these phenomena upon the calcium ion has not been investigated these speculations do not carry much weight.

The suggestion that calcium may play a role in proliferation in other tissues is certainly strengthened by the patterns of growth which have been observed in young rats (Perris et al., 1968). It was noted that the rate of growth of rats over the body weight range 60-220g was parallelled by changes in mitotic activity in bone marrow and thymus. These variations corresponded with slight increases in plasma total calcium concentrations and much more significant shifts in plasma ionised calcium. The rate of body weight gain decreased steadily

in animals weighing 220-600g, while bone marrow cell and thymocyte proliferation and plasma calcium (both total and ionised) concentrations decreased and then remained fairly steady. Younger animals tended to exhibit higher rates of cell proliferation than older rats with identical ionised calcium concentrations in the plasma, though this did not negate the obvious relationships between the parameters. Thus at different ages there could be varying sizes of calcium-responsive cell pools in the bone marrow and thymus or, alternatively, other factors might influence the sensitivity of the cells to the calcium ion. It should be noted, with regard to the latter postulate, that cells in the two tissues were responsive to calcium in mitogenic doses at all weights up to 600g (Perris <u>et al.</u>, 1968).

These disparate observations, and particularly the relationships established by Perris <u>et al.</u> (1968), suggest that calcium homeostasis may influence proliferation in a variety of body tissues, though this may not necessarily constitute a physiological control factor as in erythropoiesis and thymocyte production.

<u>5.8 Areas of Investigation.</u>

The various observations which have been exhaustively discussed above constitute a major body of evidence for the involvement of the calcium homeostatic system in the regulation of erythropoiesis and thymic lymphopoiesis. Several aspects of this regulatory role obviously required further elaboration in a mechanistic sense and the present study was therefore undertaken to clarify the following areas:

(i) Possible interrelationships between calcium homeostasis and the hormone EPO;

(ii) Elaboration of the mechanisms responsible for the restoration of red cell mass after haemorrhage;

(iii) Parallels between thymic lymphopoiesis and erythropoiesis;

(iv) The mechanism of action of the calcium ion upon bone marrow cells; and

(v) Other situations in which perturbations of extracellular calcium ion concentration might influence cell proliferation in bone marrow and thymus.

Within these broad areas of investigation, the lines of research have been, obviously, finally shaped by the observations which have been made. These observations are reported in the Results, Chapters 1 - 6, and general comments are made in the Dissertation.

GENERAL METHODOLOGY

"If we have a correct theory but merely prate about it, pigeonhole it and do not put it into practice, then that theory, however good, is of no significance."

Mao Tse-Tung.

Section 1. Introduction.

The techniques which have been used in these studies necessarily reflect the nature of the area of investigation, i.e. the inter-relationships between hormones, particularly those of the calcium homeostatic system, ions and mitotic activity in bone marrow and thymus. Thus, several methods are described for the measurement of various ions in plasma and for estimating mitotic activity in test tissues. The applicability of all methods has been rigorously examined; in particular, the repeatability and/or reproducibility of quantitative techniques has been investigated in depth.

Where a particular technique has been used only in a limited section of the work its description has been incorporated into the account of that section.

Section 2. A Note on Statistical Methods.

Values have normally been expressed as means, ± the standard error of the mean (s.e.m.) where applicable. Groups of data have been compared by use of Student's (unpaired) 't' test; values of "p" less than 0.05 ($P < 0.05$) were considered to indicate significance. Straight lines have been fitted by the method of least squares with concomitant calculation of the coefficient of correlation ("r").

Section 3. Experimental Animals.

All studies have been performed using rats of the Wistar strain. The majority of these animals were bred in the Department of Pharmacy, University of Aston in Birmingham. In some studies, Wistar rats purchased from Fisons Ltd., Bantin and Kingman Ltd., or Carworth Europe (full addresses in Appendix III) were used; no significant differences in test parameters were observed between these animals and the University-bred variety. Rats from outside suppliers were allowed a 7 - 10 day acclimation period after receipt; during this period, food and water intakes were observed to rise to steady levels, confirming the experience of Grant, Hopkinson, Jennings & Jenner (1971).

All animals, whatever their source, had been bred and maintained under constant temperature conditions. Regular (artificial) illumination schedules had not however been provided, so that experiments could not be precisely controlled to eliminate any possible seasonal effects on the parameters tested. Animals were normally maintained on a standard 41B diet (Pilsbury's Ltd.) and tap water (see Appendix II for compositions).

Groups of animals were selected for investigation by lottery from larger groups of healthy animals. The Wistar rat was found to be a convenient experimental animal because of its docility and its resistance to surgical operations and chemical treatments. However it must be remembered that its environment, where exercise is reduced and food is continually available, is essentially sedentary; to draw parallels between the response of laboratory rats and the free-living variety might therefore be invalid. Similarly, the predominantly vegetarian diet and nocturnal habits of the animal preclude many comparisons with other mammals, particularly Man. Nevertheless the elucidation of the

physiology of the Wistar rat may contribute significantly to the understanding of mammalian physiological processes in general.

Accumulated data of values of experimental parameters from normal rats are listed in Appendix I.

Section 4. Surgery.

4.1 General Considerations.

Ether anaesthesia was used throughout. Inhalation anaesthetics in general possess the advantages over injected agents of rapid induction of anaesthesia and rapid elimination from the system. Ether has particular advantages for the present studies in that it has been demonstrated to have no short-term effects on the following blood parameters: total red and white cell numbers, differential white cell counts, packed cell volume, plasma protein concentration or plasma corticosteroid levels (Grice, 1964; Besch & Chou, 1971). Furthermore, barbiturates have been demonstrated to inhibit DNA synthesis in a variety of tissues (Baserga & Weiss, 1967).

Semi-sterile technique was employed. Incision areas were shaved and swabbed with Lyseptol (Philip Harris Ltd.) and instruments and ligatures were continually soaked in one of the antiseptics. Wounds were usually closed by clips and sometimes dusted with an antibiotic. In general the rats were found to be remarkably resistant to infection, as predicted by Lambert (1965) and Ingle & Griffith (1942).

4.2 Cardiac Puncture.

The following method was routinely used for removing large volumes of blood to stimulate erythropoiesis and small volumes for analysis of plasma constituents.

Heparinised 5ml sterile syringes fitted with 21 gauge, 1.5" needles were used. The possible detrimental effects of heparin upon the accuracy of plasma ionised calcium concentration determinations are discussed later (Section 5.3). The animal was lightly anaesthetised with ether and laid on its back, tail towards the operator; the needle was then inserted into the abdomen at the midline, just caudal to the xiphisternum, and pushed slowly through the diaphragm at a low

angle into the heart. Little pressure was required to draw the plunger and fill the syringe if the entry was made cleanly into either ventricle; experience has shown that rapid withdrawal of blood against resistance may collapse one side of the heart. This puncture method was found to be superior to the intercostal method of Burhoe (1940) where difficulty was experienced in entering the heart at the first attempt. The use of ether for blood sampling procedures was mandatory, for noise stimuli can produce increases in plasma corticosteroid levels (Barret & Stockhom, 1963). Corticosteroids may affect cell viability or mitotic activity (Dougherty, 1952; Gordon, 1959; Santisteban, 1960a, b) in bone marrow and thymus.

At the termination of any experiment in which the animal had been allowed to recover after a cardiac puncture (e.g. see Results, Chapter 3) a post-mortem examination of the thoracic cavity was carried out to ensure that no internal heamorrhage had occurred. There was usually no evidence of this, but the occurrence increased in rats in which two or more cardiac entries had been made. Rats in which internal bleeding had occurred were discarded.

Sham cardiac punctures, where no blood was withdrawn, had no effect upon plasma calcium concentration or bone marrow and thymus mitotic activity; this is discussed further in Results, Chapter 3. Thus the effects of operational or anaesthetic stress were concluded to be minimal and the technique was adopted for normal use in animals where subsequent recovery was required. This method was superior to most others (e.g. tail vein, orbital puncture, decapitation) for obtaining blood for ion analysis since there was no possibility of dilution of the sample by extravascular fluids. In view of these factors the technique was considered satisfactory for routine use.

4.3 Thyroparathyroidectomy and Parathyroidectomy.

Thyroparathyroidectomy (TPTX) was performed by the method of Ingle and Griffith (1942) and parathyroidectomy (PTX) by electro-cautery. The main hazard of the operation was avoiding damage to the recurrent laryngeal nerve which usually resulted in severe respiratory difficulties.

The following routine was adhered to for both operations:

Day 1. - operation; animals allowed normal diet and tap water ad libitum.

Days 2 and 3. - normal food and tap water ad lib.

Day 4. - animals transferred to a calcium-deficient diet (Kenny & Munson, 1959) and distilled water ad libitum for the duration of the experiment. Sham-operated controls received tap water and a diet identical in all respects save that calcium was added.

Day 5. - small blood samples taken by cardiac puncture for measurement of plasma total calcium concentrations; animals in which this concentration exceeded 7mg/100ml (i.e. less than 30% lower than in normal animals) were considered to be uncertainly aparathyroid and were discarded. Values were usually in the range 4.5 - 6.5mg/100ml. The success rate was much higher in TPTX animals than in PTX.

Day 6. - required experiment performed.

Since reduction of plasma calcium concentration was taken as the criterion of successful extirpation of the parathyroid glands it was necessary to place the animals on a calcium-deficient diet, for

aparathyroid rats may partly compensate for the lack of parathyroid hormone by increasing the efficiency of calcium absorption from the gut ; this is probably a result of increased Vitamin D activity. The calcium deficient diet (see Appendix II for composition) was obtained in powder form (Scientific Products Farm Ltd.), mixed into a paste with water and baked into a form acceptable to the animals. On day 6 of the schedule food consumption had attained steady values. The animals were maintained on a calcium-containing diet for the first 48 hours after the operation to prevent any occurrence of tetany; this was never observed, despite the descriptions of parathyroid-ablated animals often encountered in classical text-books.

Although the plasma calcium concentration in the TPTX /PTX rat was the major indicator of surgical success, tremor, fur erection and thymic involution (Perris, Weiss & Whitfield, 1970) were often associated with the aparathyroid condition.

Sham TPTX was performed in the same way as TPTX except that the thyroid/parathyroid complex was not removed. The lower poles of the thyroid were cauterised in the sham PTX procedure to simulate the stimulus for the release of CT which has been observed after PTX by cautery (Hirsch, Gauthier & Munson, 1963; Costello, Stacey & Stevens, 1971).

4.4 Other Operations.

Adrenalectomy is described in Chapter 3 of Results. Hypophysectomy was performed by commercial suppliers (Carworth Europe).

Section 5. Analysis of Plasma Constituents.

5.1 Preparation and Storage of Plasma.

Blood samples were taken by cardiac puncture (see Section 4.3) in heparinised syringes. Samples were carefully ejected into glass centifuge tubes after removal of the syringe needle. This procedure prevented haemolysis. After covering with a thin layer of "oil" (liquid paraffin, light fraction), samples were centrifuged at 500Xg for 15 minutes and then stored at 4°C until required for analyses. Numerous values reported in the literature have not shown significant differences in the concentrations of serum and plasma total calcium, though this could be attributed to the great variety of analytical techniques employed. Plasma was preferredin the present studies for three reasons:

(a) Loss of (protein) clotting factors in serum might affect ionised calcium concentration;

(b) Complete clot formation requires several minutes which increases the possibility of haemolysis;

(c) The recovery of serum is not as great as that of plasma, which is an important practical consideration when dealing with small animals from which only limited volumes of blood may be obtained.

Covering samples with oil had four purposes:

(i) To reduce evaporation;

(ii) To exclude atmospheric oxygen;

(iii) To retain plasma CO_2; and

(iv) To prevent artefactual decreases in plasma total calcium concentration (see below).

Points (iii) and (iv) were the most important factors in short-

term storage. They are discussed fully in Sections 5.3 and 5.2 respectively. Plasma was easily sampled through the oil layer by Eppendorf fixed volume pipettes.

5.2 Measurement of Plasma Total Calcium Concentration.

The method used was that of Copp, Cheney & Stokoe (1963), utilising an EEL Titrator system (Evans Electroselenium Ltd.). This method was preferred to atomic absorption spectrophotometry by virtue of its speed. The accuracy was satisfactory for the present studies; this is discussed in detail below.

The principle of the method is that when calcium is complexed in alkaline solution with murexide (ammonium acid purpurate) a marked colour change occurs. Addition of ethylene diamine tetra-acetic acid (EDTA), a more powerful calcium chelating agent, to the calcium-murexide complex in graded amounts produces a gradual reversion to the original colour due to the liberation of free indicator. When all the murexide has been liberated this marks the end point of the titration, which can be accurately assessed photometrically. EDTA is added from a syringe propelled by a micrometer screw.

The end-point of the titration could be determined in two ways (manufacturer's instruction sheet):

(i) Addition of EDTA in 0.2µl aliquots until a large galvanometer movement followed by no further increase was obtained (the "visual" method) or

(ii) addition of 1µl aliquots and plotting EDTA additions against galvanometer response; the last portion of the curve forms a straight line which is extrapolated to cut the line of maximum galvanometer deflection, giving the end-point at the intersect.

Calcium standard (predicted concentration) mg/100ml	Units of EDTA titrated to end point
10	100 ± 1.4
9	86 ± 1.8
7	68 ± 1.1
5	48 ± 1.0
3	30 ± 0.7

Data plotted as Figure 6, overleaf.

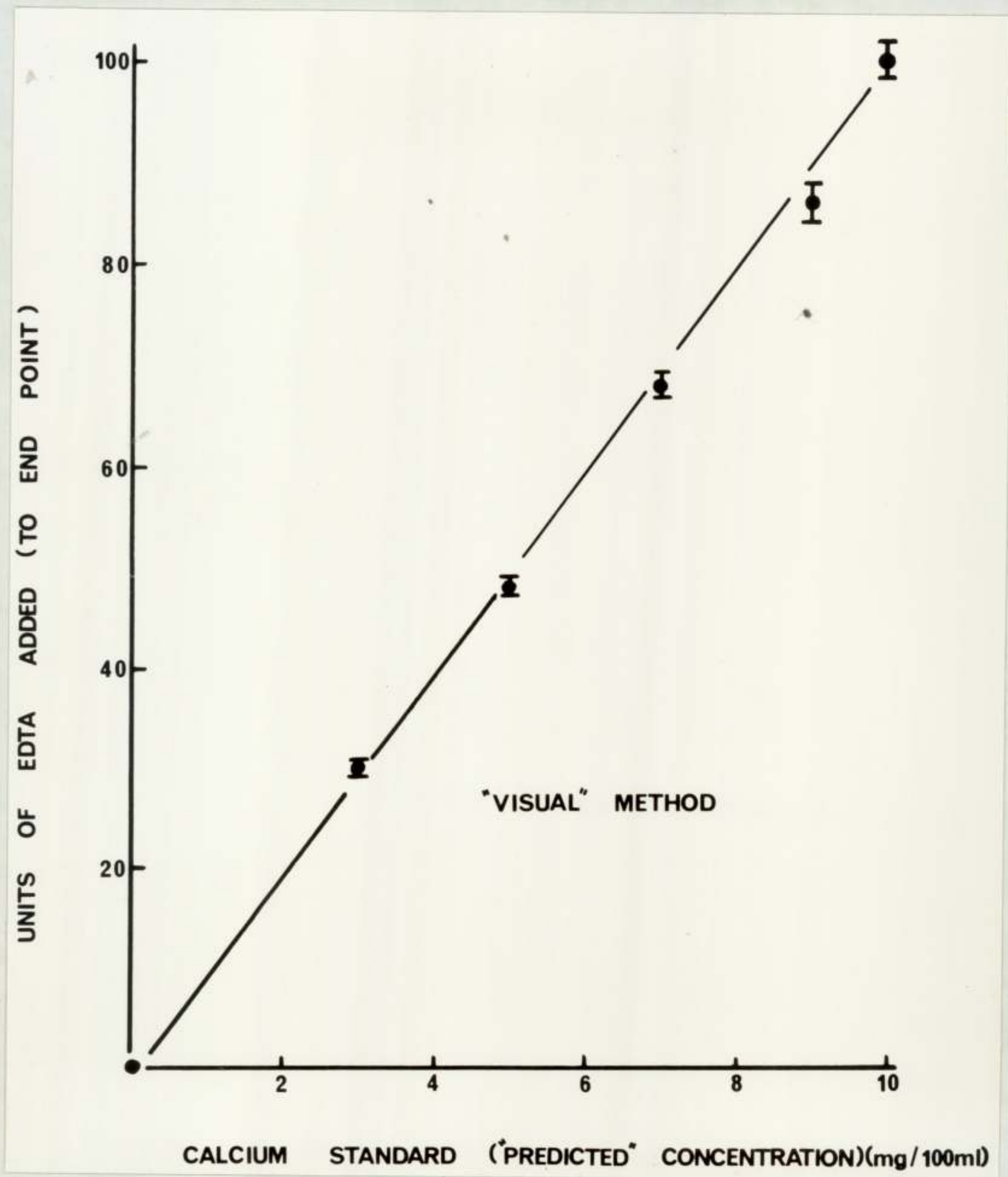


Figure 6: The "visual" method for determining the end-point of the EDTA titration against a calcium/murexide complex. Each point and bar represent the mean ± s.e.m. from 3 determinations. The line of best fit was determined by the method of least squares. 1 "unit" of EDTA = 0.2µl.

Calcium standard ("predicted" concentration) mg/100ml	Units of EDTA titrated to end point
10	100 ± 0.6
9	90 ± 0.6
7	71 ± 0.8
5	48 ± 0.5
3	29 ± 0.4

Data plotted as Figure 7 overleaf.

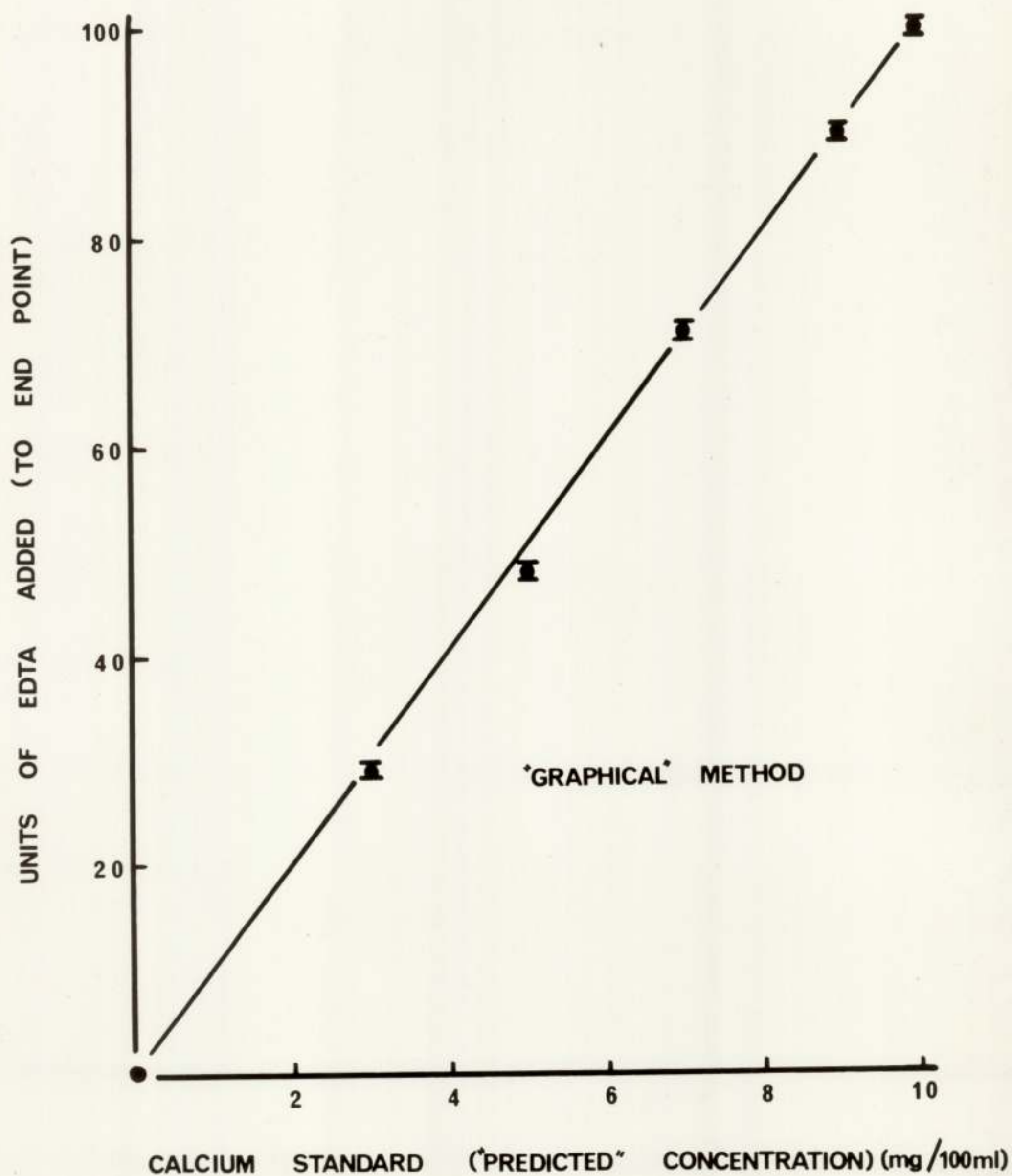


Figure 7: The "graphical" method for determining the end-point of the EDTA titration against a calcium/murexide complex. Each point and bar represent the mean ± s.e.m. from three determinations. The line of best fit was determined by the method of least squares. 1 "unit" of EDTA = 0.2µl.

Table 2 overleaf.

TABLE 2.

Plasma sample	Total calcium concentration (mg/100ml)	
	Mean ± s.e.m.	Range
1	10.1 ± 0.0	10.0 - 10.1
2	10.2 ± 0.1	10.1 - 10.3
3	10.2 ± 0.1	10.1 - 10.4
4	9.6 ± 0.1	9.4 - 9.9

Table 2: Repeatability of the technique for measurement of plasma total calcium concentration. Each sample was analysed four times to give the mean.

When a series of dilutions of a highly accurate commercial calcium standard (BDH Ltd.) were titrated against EDTA a direct linear relationship between calcium concentration and the EDTA volume added was established, using both the visual and graphical methods (Figures 6 and 7). Therefore test samples were routinely compared with a single 10mg/100ml calcium standard solution. The graphical method (Figure 7) was observed to be marginally superior to the visual method (Figure 6) for determination of the titration end-point. Comparison of Figures 6 and 7 shows that results obtained with the graphical method agree more closely with the theoretical values of 30, 50, 70, 90 and 100 "units" (1 unit = 0.2µl) of EDTA required to chelate all the calcium present, and that the spread of repeat determinations is less.

The repeatability of the method for plasma measurements was investigated. Determinations of plasma total calcium concentration were repeated four times on each of four samples in a random order (Table 2). The average standard error of the mean (s.e.m.) for each sample was less than 1% of the mean, demonstrating that the repeatability of the method was good and that the values obtained with this technique are accurate to ± 0.1mg/100ml.

Plasma from blood samples which had been centrifuged without oil coverage was compared with that from samples spun down under oil. Both total and ionised calcium concentrations were significantly lower ($P < 0.001$ in both cases) in plasma from uncovered samples than in plasma from covered samples (Table 3). This was true of all blood sample volumes between 0.5 and 3.0ml, though the values for 2ml blood samples alone are given in Table 3. Plasma phosphate, plasma total magnesium and plasma protein concentrations appeared unaffected. The methods for measurement of plasma ionised calcium, phosphate,

Table 3 overleaf.

TABLE 3.

	Plasma concentration				
	total calcium (mg/100ml)	ionised calcium (mg/100ml)	phosphate (mg/100ml)	total magnesium (mg/100ml)	protein (g/100ml)
oil covered	10.3 ± 0.20	5.45 ± 0.05	6.8 ± 0.05	2.0 ± 0.0	8.6 ± 0.2
not covered	9.2 ± 0.15	4.60 ± 0.10	6.9 ± 0.05	1.9 ± 0.1	8.7 ± 0.1
P	< 0.001	< 0.001	> 0.5	> 0.5	> 0.5

Table 3: Effect of centrifugation without oil-coverage upon plasma constituents. A number of samples were divided and centrifuged with or without coverage with light-fraction liquid paraffin. Each point is the mean ± s.e.m. from 4 samples.

Table 4 overleaf.

TABLE 4.

	Total plasma calcium concentration (mg/100ml) at:			
	0h	24h	48h	72h
Oil covered	10.3 ± 0.15	10.2 ± 0.15	10.2 ± 0.15	10.1 ± 0.10
Not covered	10.2 ± 0.2	10.25± 0.2	10.3 ± 0.15	10.4 ± 0.15

Table 4: Effects of storage with or without oil coverage at 4°C upon plasma total calcium concentration. Each point is the mean ± s.e.m. from four samples.

magnesium and protein concentrations are described in Sections 5.3, 5.5, 5.4 and 5.6 respectively.

The decrease in plasma ionised calcium was subsequently found to be independent of the presence of heparin in the sample (values not tabulated). The reduction in plasma total calcium concentration was unexpected. It could be postulated to have been a result of increased calcium binding to:

(i) red blood cells,

(ii) the glass walls of the centrifuge tube,

(iii) plasma proteins which untypically precipitated and were spun down with the red cells. This was shown not to occur (Table 3).

The effect was dependent upon centrifugation, since plasma total calcium in uncovered tubes does not decrease further during subsequent storage (Table 4). The postulated changes in the binding of calcium could have been a result of loss of CO_2 from the plasma and the consequent increase in pH; in normal plasma, increases in pH produce changes in calcium binding to protein (Toribara, Terepka & Dewey, 1957; Loken, Havel, Gordan & Whittington, 1960; Prasad, 1960; Moore, 1970). Note that plasma phosphate and magnesium concentrations were not affected by centrifugation when uncovered (Table 3). The above hypotheses have not been investigated. However, in subsequent experiments blood samples were oil-covered prior to centrifugation to prevent invalidation of experimental results by these gross changes in plasma calcium concentrations (Table 3).

It was established that blood samples could be stored at 4°C for up to 72h after centrifugation without significant changes in total calcium concentration in the plasma, with or without oil coverage (Table 4). The uncovered samples investigated had been centrifuged under oil which was subsequently removed.

It is extremely unlikely that oil contamination could have produced abnormally high plasma calcium concentrations. Liquid paraffin has no appreciable calcium content, and the values for plasma total calcium concentration were similar to those quoted by a number of research publications and reference data books.

The method was considered suitable for routine use within the limitations discussed above.

5.3 Measurement of Plasma Ionised Calcium Concentration.

Calcium exists in plasma in three forms (Rona & Takahashi, 1913; Marrack & Thacker, 1926; Greenberg & Gunther, 1930; Nicholas, 1932; Watchorn & McCance, 1932; Dillman & Visscher, 1933; McLean & Hastings, 1934, 1935; Morrison, McLean & Jackson, 1938; Neuman & Neuman, 1958; Prasad & Flink, 1958; Breen & Freeman, 1961; Moore, 1970):

(i) non-diffusible, protein bound calcium (30 - 50% of the total, depending upon pH and temperature);

(ii) diffusible, non-ionised calcium (complexes with small molecules, comprising about 15% of the total); and

(iii) ionised calcium.

It has long been established that the ionised form is the physiologically active species (McLean & Hastings, 1934, 1935; Toribara et al. 1957) and many important physiological processes are known to be critically dependent upon calcium ion activity (see General Introduction, Section 4). Previous methods for the direct measurement of ionised calcium concentration in biological fluids — frog heart (McLean & Hastings, 1934, 1935), rachitic rat cartilage (Yendt, Connov & Howard, 1955), ultrafiltration (Morrison et al. 1938) and metal ion indicators (Walser, 1960) — have been shown to be indirect, imprecise and time consuming (Pittinger, 1970). The development of an electrode specific for the calcium ion has progressed from the

original, unsatisfactory glass electrode (Truesdell & Christ, 1967) through a dip-type ion-exchange electrode (Ross, 1967) to the present flow-through ion-exchange system developed specifically for measurements of ionised calcium in plasma and serum (Moore, 1969, 1970).

The equipment used in this study was an Orion 99-20 flow-through electrode (Orion Research Inc.) coupled with an Orion Model 801 digital mV/pH meter. The advantages of the flow-through system over the older dip-type electrode have been described by Moore (1970). Briefly, they are:

(i) more rapid equilibration;

(ii) greater stability of potential;

(iii) smaller sample volume requirement; and

(iv) anaerobic conditions for measurement, which maintain blood pH by preventing CO_2 loss.

The principle of the ion-exchange electrode is that a chemical exchange of calcium for another ion occurs at the interface of a test solution and an organic ion-exchange resin, thereby generating a potential. Thus the electrode measures calcium ion <u>activity</u> and not calcium ion concentration. However, in dilute solutions of calcium such as biological fluids the activity of the ion is proportional to its concentration and it is therefore possible to calibrate the electrode in terms of ionised calcium concentration (Moore, 1970). The relationship between the ion activity and electrode potential (Ross, 1967) is given by:

$$E = Ea + 2.3 \cdot \frac{RT}{nF} \cdot \log A \qquad (1)$$

where E = measured potential

Ea = fraction of potential determined by choice of reference electrodes and internal solutions.

$2.3 \cdot \frac{RT}{nF}$ = Nernst factor

A = activity of the ion in the sample.

Thus where the temperature and choice of electrodes, etc., are constant for all determinations the measured potential is directly proportional to the activity of the calcium ion.

The system was operated in accordance with the manufacturer's instructions. A standard curve was established before each series of measurements. The standard solutions (Orion Research Inc.) contained 0.5, 1.0 or 2.0 x 10^{-3}M (2, 4 or 8mg/100ml) $CaCl_2$ and 1.5 x 10^{-3}M NaCl. The sodium content of the standard solutions approximates the sodium concentration of plasma and therefore equates for errors produced by sodium interference in electrode response for samples of both plasma and standard. Small quantities of trypsin and triethanolamine were added to the standards when the system was first used. Triethanolamine was recommended by the manufacturer to retain electrode membrane flexibility; the function of trypsin was to clear possible deposits of plasma proteins which might precipitate on the membrane. Although addition of these substances causes a small error in electrode response (Li & Piechocki, 1971; Lindgarde & Zettervall, 1971; Schwartz, McConville & Christopherson, 1971) several authors (Hattner, Johnson, Berstein, Wachman & Bracknam, 1970; Li & Piechocki, 1971; Lindgarde & Zettervall, 1971) have claimed that both additives are necessary to preserve membrane stability and precision. However, it was observed in operation that a white deposit accrued upon the membrane and since this was markedly reduced when trypsin was omitted from the standard solutions this deposit may have been the enzyme itself. Omission of trypsin was found to increase membrane life without noticeably affecting membrane response. The enzyme was therefore dispensed with in subsequent work. Since plasma samples do not contain either of the additives it is clear that comparison of plasma ionised concentrations with those of standard solutions containing triethanolamine, trypsin or both, could produce

Table 5 overleaf.

TABLE 5.

Plasma sample	Ionised calcium concentration (mg/100ml)	
	Mean ± s.e.m.	Range
1	5.70 ± 0.0	5.70 - 5.75
2	5.60 ± 0.0	5.55 - 5.60
3	5.60 ± 0.05	5.60 - 5.65
4	5.55 ± 0.0	5.55 - 5.60
5	5.30 ± 0.05	5.25 - 5.30
6	5.50 ± 0.05	5.45 - 5.55
7	5.80 ± 0.05	5.75 - 5.90
8	5.55 ± 0.05	5.50 - 5.55

Table 5: Repeatability of the flow-through ion-exchange method for measurement of plasma ionised calcium. The mean was derived from 4 determinations.

erroneous results if the additives affect electrode response. One approach to this problem would be to dissolve additives in the test plasma. However, this is obviously impractical since the minute quantities necessary in small plasma samples would be impossible to administer. Therefore the procedure of Schwartz et al. (1971) was adopted. After membrane stability had been achieved with standards containing triethanolamine, standards without this additive were analysed. A comparison of the two calibration lines thus obtained revealed that the treated standards gave a decreased electrode response. The "true" ionised calcium concentrations of the treated standards were determined and plasma samples were then compared with the calibration line plotted using these "true" values. The decrease in ionised calcium concentration in the treated standards is probably due to complexing of the ion (Schwartz et al., 1971).

Standards were routinely flushed through the system for 60 minutes to allow the electrode to achieve stability prior to calibration. Thereafter, 0.2ml plasma samples could be run through in groups of four. After each such group the median (1mM) standard solution was used to clear the system and to check whether the electrode response was "creeping".

The electrode was operated only when the difference between the 1mM and 2mM calcium standards was 7.5mV or greater; below this value there was a concomitant loss of precision as the slope of the calibration curve decreased. Freshly-prepared electrodes showed a difference of 7.8 - 8.6mV between the 1mM and 2mM standards. Thus the smallest change in potential which could be detected, $\pm$ 0.1mV, was equivalent to approximately $\pm$ 0.05mg ionised calcium/100ml.

Multiple determinations of ionised calcium concentrations in single plasma samples confirmed that the repeatability of the method

Table 6 overleaf.

TABLE 6.

Temperature (°C)	Plasma ionised calcium concentration (mg/100ml)
4	5.40 ± 0.10
21	5.30 ± 0.10
37	5.25 ± 0.05

Table 6: Effect of sample temperature upon measurement of plasma ionised calcium by flow-through ion exchange system. Values are means ± s.e.m. from four samples.

Table 7 overleaf.

TABLE 7.

	Plasma ionised calcium (mg/100ml) at:		
	0h	24h	48h
Oil covered	5.35 ± 0.10	* 5.35 ± 0.10	5.25 ± 0.10
Not covered	5.30 ± 0.05	* 4.80 ± 0.15	–

Table 7: Effect of storage with or without oil-coverage at 4°C upon plasma ionised calcium concentration. * $P < 0.01$.
Each point is the mean ± s.e.m. from four samples.

was satisfactory (Table 5; see also Li & Piechocki, 1971). The average s.e.m. for four determinations from a sample was less than 1% of the mean. All measurements were taken at ambient temperature (18 - 24°C) in a room sheltered from draughts and electrical interference. Some authors (Truesdell & Christ, 1967; Hansen & Theodorsen, 1971) have suggested that sample temperature affects electrode response; this would be expected, since the Nernst factor if temperature dependent. Separate aliquots of plasma samples were measured at 4°C, 21°C and 37°C (Table 6). The difference between the 21°C samples and those at the other temperatures was approximately 1.5% which is in good agreement with the value obtained by Hansen & Theodorsen (1971). Thus although detectable ionised calcium decreases with increasing temperature over the 4°C - 37°C range (see Table 6), any possible errors caused by changes in ambient temperature were considered negligible. Note that equation (1) would predict an increase in electrode response with increasing temperature if the ion activity remained constant. Thus the temperature rise probably increased the binding of calcium to protein (Hansen & Theodorsen, 1971).

No changes occurred in ionised calcium concentration in plasma samples which had been stored under oil for up to 48 hours at 4°C (Table 7). However, uncovered samples exhibited a significant decrease in ionised calcium concentration over a 24 hour period at 4°C (Table 7). Recall that plasma total calcium concentration did not decrease under identical storage conditions over the same time period. Presumably this decrease in ionised calcium concentration was due to changes in calcium binding to plasma protein resulting from pH changes following CO_2 loss from the plasma. Loss of CO_2 causes an increase in pH, according to the Henderson-Hasselbach equation:

Plasma heparin concentration (I.U./ml)	Plasma ionised calcium concentration (mg/100ml)
0	4.9
1	4.9
2	4.85
10	4.7
25	4.3
50	2.2

Data plotted as Figure 8 overleaf.

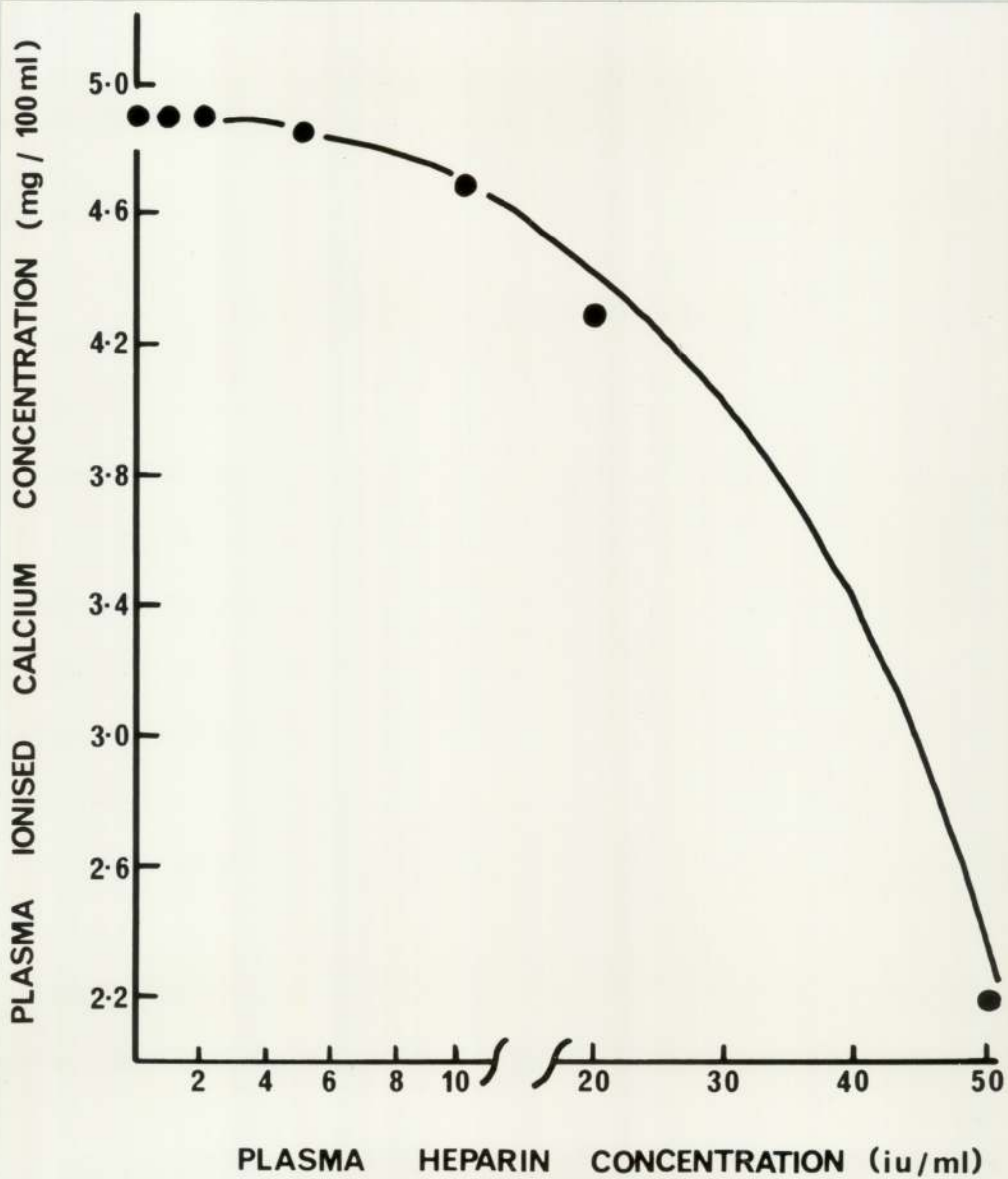
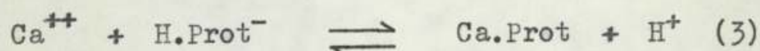


Figure 8: Effect of heparin upon plasma ionised calcium determination. Each point represents the mean from two plasma samples.

$$pH = pK + \log. \frac{HCO_3^-}{H_2CO_3} \qquad (2)$$

Changes in pH produce changes in calcium binding protein, governed by the equilibrium:



$$Ca^{++} + H.Prot^- \rightleftharpoons Ca.Prot + H^+ \quad (3)$$

All blood samples were drawn in heparinised syringes. As heparin has some calcium-chelating ability it was necessary to check whether heparin, as thus used, could significantly affect the ionised calcium fraction in plasma or interfere with its measurement by the ion-exchange electrode. Normal heparin concentration was approximately 1i.u./ml of whole blood. Various multiples of this amount were added to aliquots from single blood samples; after centrifugation, plasma ionised calcium was measured. Although high concentrations of heparin produced dramatic falls in measured free calcium (Figure 8), normal concentrations had no effect; at least a ten-fold increase in heparin concentration in the plasma was required to produce a reduction of 0.2mg/100ml in plasma ionised calcium concentration (i.e. approximately a 4% decrease).

The method was therefore concluded to be satisfactory for measurements of ionised calcium concentration in heparinised plasma samples if care was taken to maintain pH; the maximum precision was considered to be $\pm$ 0.05mg/100ml.

5.4 Measurement of Plasma Magnesium Concentration.

Two methods for measuring plasma magnesium concentration were investigated; EDTA titration, utilising Eriochrome Black T as indicator, and atomic absorption flame spectrophotometry. The former was found to be inaccurate and only poorly reproducible and the latter

Magnesium concentration (mg/100ml)	Transmittance (%)
0	100
0.5	89.8 ± 0.1 (8)
1	79.8 ± 0.8 (6)
2	61.8 ± 0.9 (8)
4	28.6 ± 2.0 (4)

Data plotted as Figure 9 overleaf. Number of determinations given in parentheses.

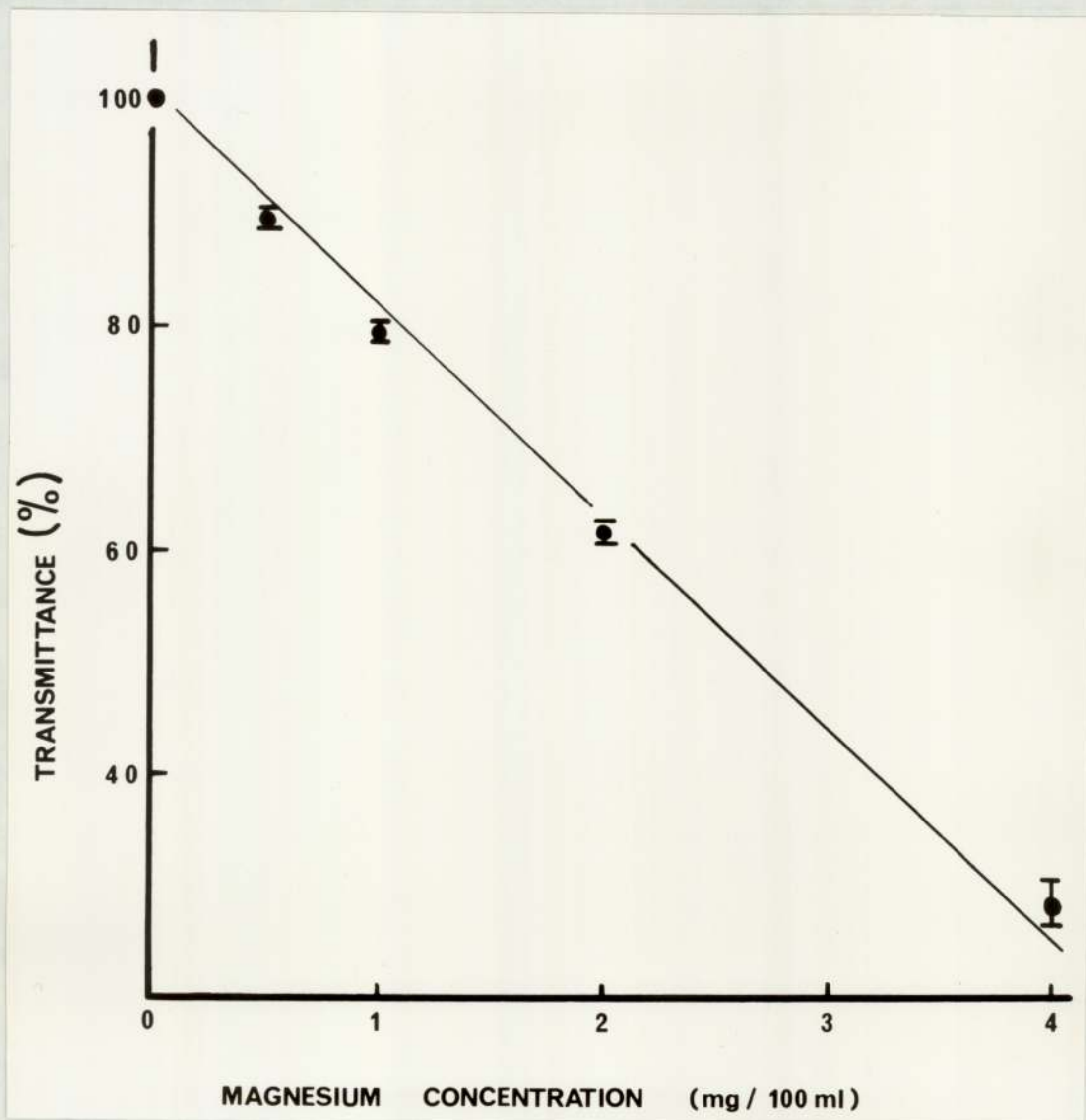


Figure 9: Standard curve for determination of magnesium concentration by atomic absorption flame spectrophotometry. Each point and bar represent the mean ± s.e.m. from 4 - 8 determinations. Line of best fit calculated by method of least squares; r = 0.98.

Table 8 and Table 9 overleaf.

TABLE 8.

Plasma sample	Total magnesium concentration (mg/100ml)	
	Mean ± s.e.m.	Range
1	2.1 ± 0.0	2.1 - 2.2
2	2.1 ± 0.1	1.8 - 2.2
3	1.6 ± 0.0	1.5 - 1.6
4	2.0 ± 0.05	1.9 - 2.1

Table 8: Repeatability of the method for measurement of plasma magnesium concentration. Mean derived from 4 determinations.

TABLE 9.

Plasma sample	Total magnesium concentration (mg/100ml)	
	Mean ± s.e.m.	Range
1	1.9 ± 0.1	1.6 - 2.1
2	2.0 ± 0.05	1.9 - 2.1
3	2.2 ± 0.1	1.8 - 2.3

Table 9: Reproducibility of the method for measurement of plasma magnesium concentration. Samples were divided into aliquots prior to deproteinisation. Mean derived from 4 aliquots.

was adopted for routine use. Note that "plasma magnesium concentration" refers to the total concentration of the metal in plasma; the ionised fraction was not determined separately.

Plasma samples were diluted twenty-fold and deproteinised with trichloroacetic acid in the presence of 2×10^3 p.p.m. strontium chloride; strontium reduces the interference of other ions, particularly phosphate, in solution (Murdoch & Heaton, 1968). After centrifugation at 350 x g for 5 minutes the supernatant was analysed in a Unicam SP900A atomic absorption flame spectrophotometer. The flame was an acetylene/air mixture. Wavelength was 285.2mμ, slit-width 0.09 - 0.12mm and lamp current 8mA. Samples were read in comparison with a blank solution and a magnesium standard prepared from a commercial standard (BDH Ltd.). Strontium and trichloracetic acid were added to these solutions in the same proportion as to the plasma samples. The standard curve for the normal range of magnesium concentration in plasma is shown in Figure 9. Transmission was directly proportional to sample concentration over the limited range studied.

A decrease in transmittance of 2 units was approximately equivalent to 0.05mg/100ml magnesium; however it was considered that the minimum detectable difference between samples was ± 0.1mg/100ml. The repeatability and reproducibility of the method were examined (Tables 8 and 9). The s.e.m. of four replicate determinations on single samples averaged approximately 2% of the mean, showing the repeatability to be good. A single plasma sample was divided into four prior to deproteinisation and the magnesium concentration was measured in each aliquot; the s.e.m. was approximately 5% of the mean, showing the reproducibility of the technique to be reasonable. As shown in Table 3, plasma magnesium concentration was unaffected by centrifugation without oil coverage.

Phosphate concentration (mg/100ml)	Absorbance
0	0
1	0.037 ± 0.002 (8)
2	0.074 ± 0.005 (8)
4	0.105 ± 0.002 (12)
8	0.212 ± 0.002 (12)

Data plotted as Figure 10 overleaf. Number of determinations given in parentheses.

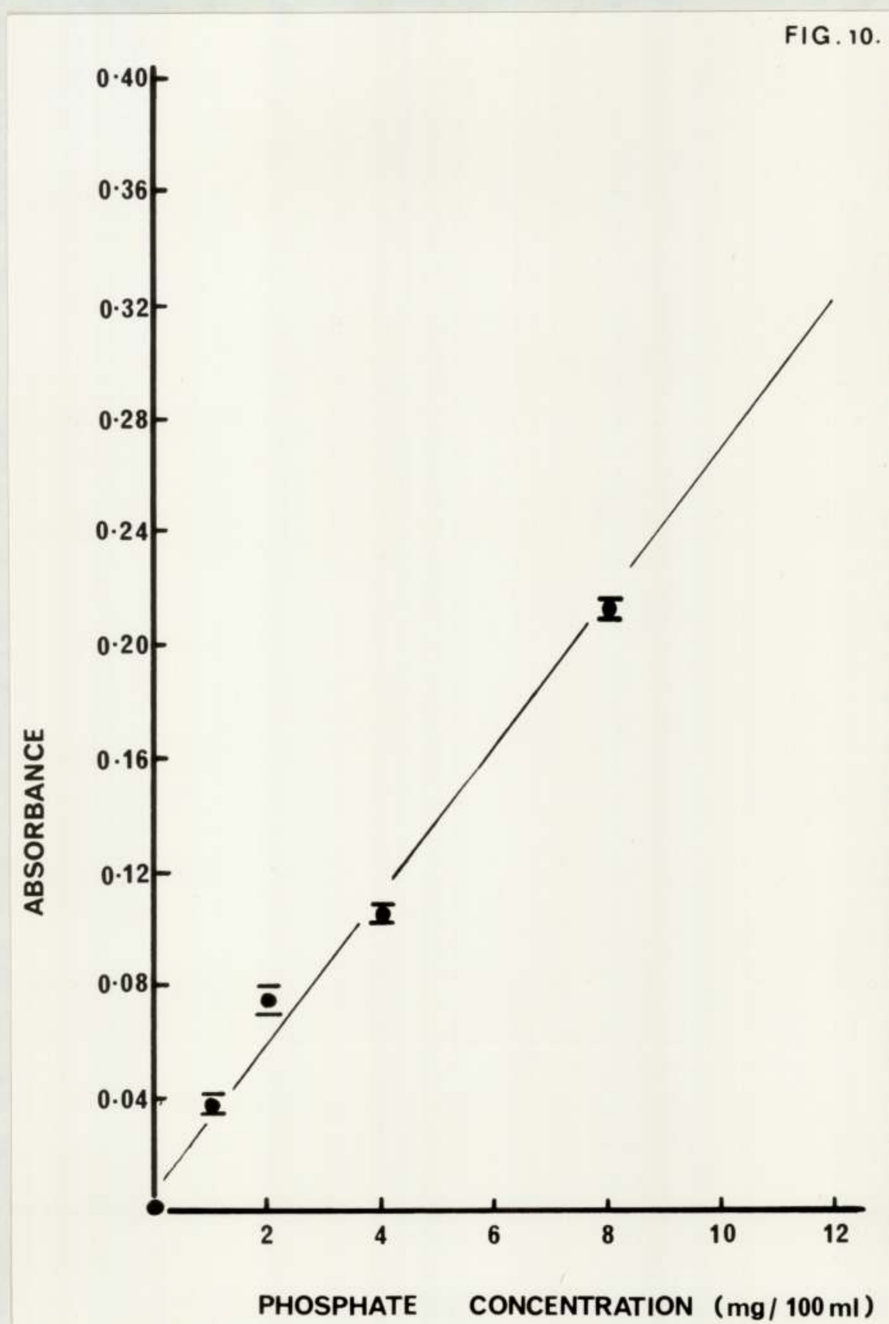


Figure 10: Standard curve for determination of phosphate concentration. Each point represents the mean ± s.e.m. derived from 8 - 12 determinations. Line of best fit by method of least squares; r = 0.97.

Table 10 and Table 11 overleaf.

TABLE 10.

Plasma sample	Phosphate Concentration (mg/100ml)	
	Mean ± s.e.m.	Range
1	6.35 ± 0.1	6.2 - 6.5
2	6.8 ± 0.1	6.7 - 7.1
3	7.7 ± 0.1	7.4 - 7.9
4	6.8 ± 0.0	6.7 - 6.9

Table 10: Repeatability of the method for measurement of plasma phosphate concentration. Mean derived from 4 determinations.

TABLE 11.

Plasma sample	Phosphate Concentration (mg/100ml)	
	Mean ± s.e.m.	Range
1	6.8 ± 0.2	6.4 - 7.7
2	6.9 ± 0.05	6.7 - 7.0
3	7.3 ± 0.2	6.8 - 7.7

Table 11: Reproducibility of the method for measurement of plasma phosphate concentration. Each plasma sample was split into seperate aliquots prior to deproteinisation. Mean derived from 4 aliquots.

The method was therefore adopted for measurement of plasma magnesium concentration; the limit of precision was considered to be ± 0.1mg/100ml.

5.5 Measurement of Plasma Inorganic Phosphorus Concentration.

The terms "plasma inorganic phosphorus" and "plasma phosphate" are often used interchangeably by research workers though they are obviously not completely synonymous. The method of Chen, Toribara & Warner (1956) is often stated to measure plasma phosphate concentrations though it actually measures inorganic phosphorus. In the present study the term phosphate is used with acknowledgement of the assumption that essentially all inorganic phosphorus is present in plasma as phosphate. Thus concentrations of phosphate which are quoted here are actually concentrations of inorganic phosphorus.

After deproteinisation with trichloroacetic acid, plasma phosphate concentrations were measured by the method of Chen, Toribara and Warner (1956). Absorbance was read against a blank at 820mμ in a Unicam SP500 spectrophotometer. Standard solutions were prepared from potassium dihydrogen orthophosphate. The standard curve for phosphate determinations in the normal range for plasma is shown in Figure 10. A change in absorbance of 0.03 units was equivalent to 0.1mg/100ml phosphate; this was considered to be the minimum detectable difference between two samples.

Repeat readings of single samples showed the repeatability of the method to be satisfactory (Table 10); the average s.e.m. was less than 1% of the mean. The reproducibility of the method was investigated (Table 11) as for magnesium (Section 5.4); the s.e.m. was approximately 3% of the mean. As shown previously (Table 3), centrifugation of blood samples without oil-coverage did not affect plasma phosphate concentrations.

Tables 12 and 13 overleaf.

Sample	Plasma protein concentration (g/100ml)	
	Mean ± s.e.m.	Range
1	8.5 ± 0.0	8.4 - 8.6
2	8.8 ± 0.1	8.6 - 9.0
3	7.6 ± 0.0	7.5 - 7.7
4	8.6 ± 0.1	8.5 - 8.8
5	9.1 ± 0.1	8.7 - 9.2

Table 12: Repeatability of the Biuret method for measurement of plasma protein concentration.

Sample	Plasma protein concentration (g/100ml)	
	Mean ± s.e.m.	Range
1	8.6 ± 0.1	8.5 - 8.8
2	7.3 ± 0.1	7.0 - 7.5
3	8.1 ± 0.2	7.6 - 8.3
4	9.0 ± 0.1	8.8 - 9.2

Table 13: Reproducibility of the Biuret method for measurement of plasma protein concentration.

The method was adopted for routine measurement of plasma phosphate concentration and $\pm$ 0.1mg/100ml was considered to be the limit of precision.

5.6 Measurement of Plasma Protein Concentration.

The method of Lowry and his associates (Lowry, Rosebrough, Farr & Randall, 1951) was unsuitable for measurement of plasma proteins without prior dilution of the sample. For simplicity, and to obviate the introduction of a further source of experimental error (i.e. the dilution procedure), the Buiret method of Gornal, Bardawill & David (1949) was investigated and then adopted.

The protein solution (in this case plasma) is treated with the cupric ion in alkaline solution to yield a violet complex. The intensity of the colour is directly proportional to the original protein concentration. The colour reaction was allowed to proceed for 30 minutes at room temperature and the samples were then read against a blank at 540mμ on an SP600 spectrophotometer. The absorbance was compared with that of a standard protein solution (10g/100ml) prepared from highly purified ovine albumin powder (Sigma Chemicals).

A standard curve was constructed and found to be linear over the range 0 - 12g/100ml. The repeatability and reproducibility of the method were assessed (Tables 12 and 13) and found to be satisfactory. Repeat readings of single samples over a 30 minute period yielded a mean with a s.e.m. of approximately 1.5% of the mean value (Table 12). The reproducibility of the method was investigated (Table 13) as before (Sections 5.4), and the s.e.m. found to be 3% of the mean. As mentioned previously (Table 3), centrifugation of blood samples did not appear to affect plasma protein concentrations.

A 10g/100ml protein solution was found to produce an absorbance of approximately 0.24 by this method. Since 0.002 absorbance units was the limit of discrimination of the absorbance scale it was concluded, taking into account the data on repeatability and reproducibility in Tables 12 and 13, that the limit of resolution of the method was ± 0.1g protein/100ml plasma. Since plasma protein concentrations were determined directly after sampling, data on effects of storage were not collected.

Section 6: Estimation of Mitotic Activity in Bone Marrow and Thymus.

6.1 General Considerations.

Mitotic activity has been assessed by two morphological methods:

(i) determination of mitotic index in the tissues or

(ii) use of the metaphase-arresting alkaloid colchicine (BDH Ltd.) or its derivative demecolcine (Colcemid, CIBA Ltd.).

The methods are described in detail in subsequent sections (6.2 to 6.5). In brief, the general approach is to prepare cell suspensions which are smeared, stained and scored for the percentage of nucleated cells present in mitosis (the mitotic index) or the percentage of nucleated cells arrested in the metaphase stage of mitosis after prior treatment with colchicine or Colcemid.

6.2 Preparation of Smears.

Bone marrow and thymus tissues were removed from animals which had been lightly anaesthetised with ether.

The right femur was removed, the diaphyses were cut off and the marrow plug extruded into 1ml of 0.9% (0.154M) saline. The tissue was dispersed in the medium by gentle aspiration with a fine Pasteur pipette; frothing was avoided as this might have caused cell damage due to surface tension effects. Two drops of this suspension were mixed with a single drop of inactivated calf serum (Wellcome Reagents Ltd.) on a slide, thinly smeared and dried at 37°C in an air stream.

The whole thymus was removed, washed in 0.9% saline and minced with surgical scissors in 1ml of the same medium; the suspension was filtered through four layers of dampened cheese-cloth and dispersed in a further 2ml of saline. One drop of the final suspension was mixed with two drops of calf serum on a slide and smeared and dried as for bone marrow.

Physiological (0.9%) saline was an adequate medium for maintaining cell morphology for short periods. The cells were fixed in neutral 10% formalin and stained with Delafield's haematoxylin. To minimise cell damage the time between removal of the tissue from the animal and fixation was kept as short as possible. Cells undergoing division may be more fragile than non-dividing cells (Lala, Maloney & Pratt, 1964); thus a long delay prior to fixation could artificially reduce the percentage of dividing cells in the smear, in addition to making cell identification difficult.

6.3 Mitotic Index.

The measurement of mitotic index is best discussed in the light of the cell cycle as a whole. A diagram of the cell cycle (Howard & Pelc, 1953) is shown in Figure 5; p. 78):

"G_1" is the post-mitotic gap period, in which the cell contains a diploid (2n) quantity of deoxyribonucleic acid (DNA).

"S" is the period of DNA synthesis, in which the cell may contain amounts of DNA varying between the diploid (2n) and the tetraploid (4n) complement.

"G2" is the pre-mitotic gap period, in which 4n quantity of DNA is present in the cell.

"M" is the mitotic period.

"G_0" is the phase in which mitotically competent diploid cells are quiescent; these cells can be recruited into the cycle, probably at the G_1/S boundary (Whitfield et al., 1969a, b).

"G_2^1" is another "resting" phase in which cells with a 4n quantity of DNA are poised on the boundary of mitosis. These cells can be recruited directly into the early part of the M phase. This population is chiefly of significance in skin epidermal cells; a number of mitogenic hormones and ions pertinent to the present study have been shown not to exert their effect by actions upon cells in G_2 (Whitfield et al., 1969a).

"Inert" cells probably do not enter the cell cycle though it may not be justifiable to deem them incapable of doing so, or of exchanging

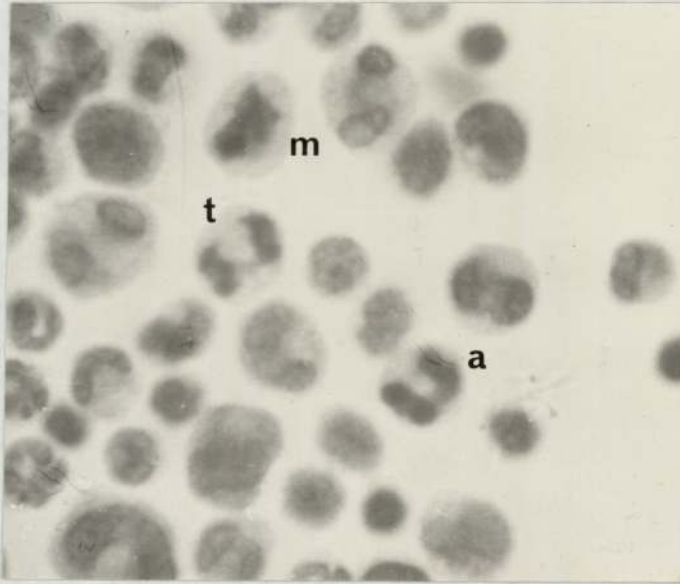
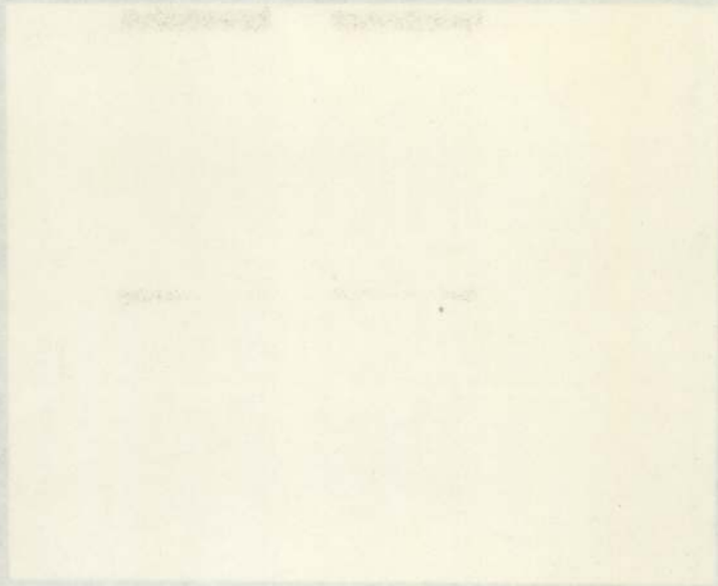


Plate 1: Mitotic figures in bone marrow cells (approx. x 1600).
m = late metaphase; a = anaphase; t = late anaphase/
early telophase.



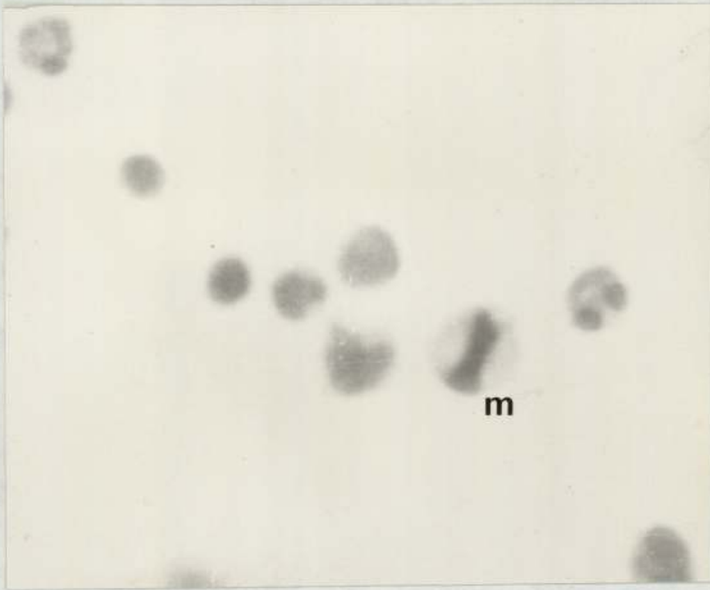


Plate 2: Normal metaphase figure (m) and non-dividing bone marrow cells (approx. x 1500).

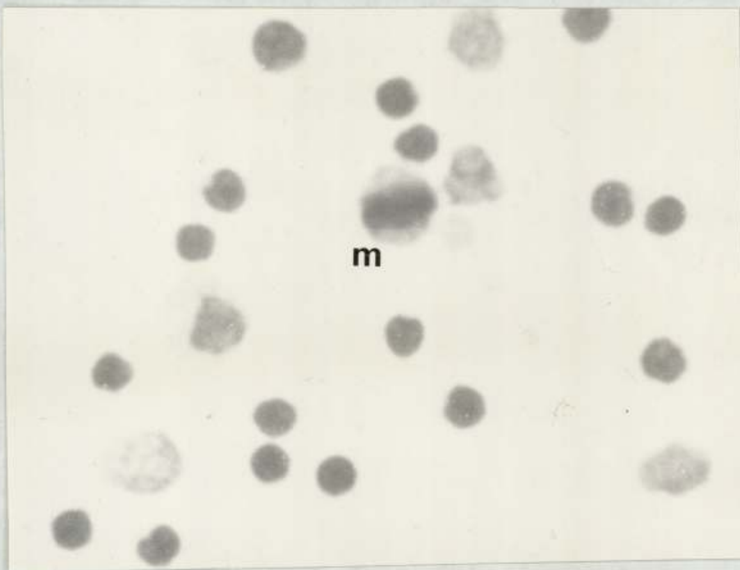


Plate 3: Late normal metaphase/ early anaphase (m) and non-dividing thymocytes (approx. x 1500).

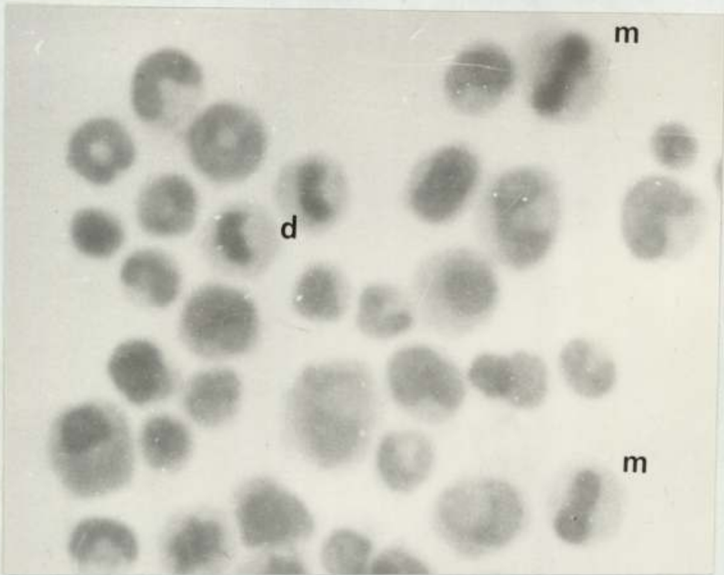


Plate 4: Mitotic figures in bone marrow cells (approx. x 1600).

m = normal meta-hase; d = freshly divided cells.

with the G_0 population, under certain circumstances.

The "four-phase" cell cycle ($G_1 \rightarrow S \rightarrow G_2 \rightarrow M$) of Howard & Pelc (1953) has been found to pertain to almost all plant and animal cells. There are some exceptions (Ord, 1973) which are not relevant to the present study.

The mitotic index is classically defined as the percentage of nucleated cells present which are in any stage of mitotic progression (prophase → metaphase → anaphase → telophase). Late prophase, metaphase, anaphase and telophase figures were easily identifiable (see Plates 1 - 4); however, very early prophase figures were not readily distinguishable from non-dividing cells. It was therefore necessary in this study to define mitotic index as the percentage of nucleated cells present which were in any stage of mitosis from late prophase to the end of telophase.

Increases in mitotic index could be a consequence of several factors:

(i) The destruction or extrusion of large numbers of mitotically incompetent non-cycling cells (e.g. small thymocytes) from the tissue. This would artificially enrich the percentage of the remaining, mitotically competent, nucleated cells which was undergoing mitosis without affecting the absolute numbers of cycling cells. To produce a 50% increase in mitotic index in the bone marrow (say, from 1.2 to 1.8) in this fashion would require a 33% elimination of mitotically incompetent nucleated cells. Changes in mitotic index of this magnitude have frequently been observed in the present study and it seems most unlikely that such an enormous cell loss could be responsible. Indeed it has previously (Perris & Whitfield, 1971) been shown that increases in bone marrow mitotic index after injections of the calcium ion are certainly not associated with any reduction in the numbers of non-dividing cells. Furthermore, a number of hormones and ions which increase bone marrow and thymus mitotic index are also active <u>in vitro</u>

(Results Chapters 5 and 6; Perris, 1971; Whitfield et al., 1973c) which would preclude the possibility of nucleated cell extrusion, though not that of destruction.

The evidence therefore suggests that the changes in mitotic activity in the test tissues which have been observed in a number of situations in the present studies could not be ascribed to the phenomenon described above, i.e. decreases in non-dividing nucleated cells without concomitant decreases in cells undergoing mitosis.

(ii) Prolongation of the mitotic period, M, without a corresponding increase in the cell cycle time as a whole. Thus the proportion of the cell cycle occupied by dividing cells would be increased, for although cells would enter mitosis at the normal rate the prolongation of their stay in this phase would lead to an increase in mitotic index which could be misinterpreted as an increase in mitotic activity. Parallel measurements of the true rate of entry of cells into M using the colchicine metaphase accumulation technique (see Section 6.4 for detailed discussion) would preclude such erroneous conclusions.

(iii) A shortening of the overall cell cycle time without any decrease in the mitotic period would also increase the proportion of cycling cells in mitosis at any one time. Such a situation could truly be interpreted as an increase in the rate of production of new cells. Although changes in overall cell cycle time in haemopoietic tissues almost certainly exist (Tarbutt, 1969), the portion of the cell cycle which is affected is not clear. Decreases in the length of the G_1 phase relative to the rest of the cycle have only been demonstrated unequivocally to be of significance in tissues not relevant to the study of haemopoiesis and lymphopoiesis in the adult rat, e.g. rapidly dividing cells in some early embryos or tissue culture lines, some fully differentiated tissues, certain tumour cells, and in Amoebae and yeasts (Ord, 1973). No changes in overall cell cycle time without concomi-

tant changes in the mitotic period have been demonstrated in bone marrow or thymus cells. Indeed, Alpen & Cranmore (1959) have concluded that the G_1 phase is lacking or invariable in erythroblasts. Furthermore, since the G_2 and S phases of the cycle appear to be relatively constant under most conditions (Cameron, 1970), it is clear that large decreases in the duration of the G_1 phase would have to be postulated to explain changes in mitotic index of 50% (which have been observed in many experimental conditions). To use again the figures quoted above (p.124), an increase in mitotic index from 1.2 to 1.8 would require an overall reduction of 33% in the cycle time with M remaining constant. However, in a number of rapidly proliferating mammalian tissues, such as rat duodenal, ileal and jejunal crypts, mouse and hamster jejunal crypt, canine erythroid and myeloid precursors and bovine lymphocytes (all quoted by Cameron, 1970) the G_1 phase constitutes only 10 - 20% of the total cycle time. Thus, although it must be acknowledged that the tissues quoted above do not directly apertain to the studies at hand, it seems likely that change in the overall cycle time without concomitant change in M is not a significant factor in the production of variations in mitotic index in the tissues which have been examined in the present study.

(iv) Recruitment of cells from the (resting) G_0 population while cell cycle time and total nucleated cell numbers remain essentially constant. Thus an increased percentage of the total nucleated cell population would truly be engaged in proliferative activity. This possibility has been postulated to account for the increases in mitotic index reported in the present investigations (Results, Chapters 1, 2, 3, 4, and 6).

Decreases in mitotic index could similarly be explained by the obverse of the above hypotheses.

Hours after initial Colemid injection	% nucleated cells in metaphase
2	5.3 ± 1.0 (4)
4	12.4 ± 0.9 (12)
6	15.8 ± 0.6 (18)

Data plotted as Figure 11, overleaf. Number of animals given in parentheses.

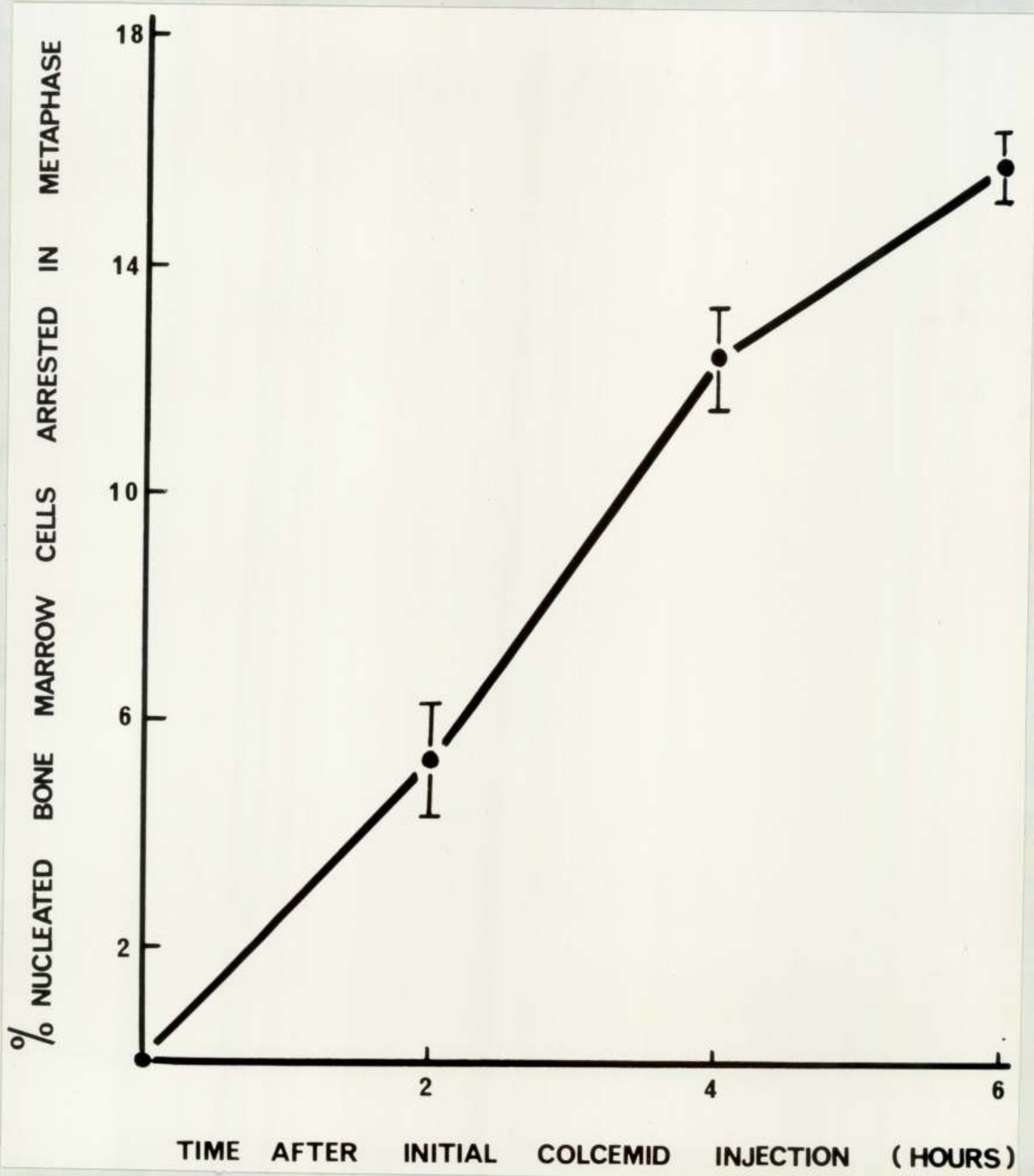


Figure 11: Linear progression of bone marrow cells into metaphase after Colcemid treatment <u>in vivo</u>. Colcemid (0.2mg/100g body weight) injected at 0 and 3h. Each point and bar represents the mean ± s.e.m. derived from 4 - 18 animals.

Hours after initial Colcemid injection	% nucleated cells in metaphase
2	2.7 ± 0.6 (4)
4	5.6 ± 0.5 (14)
6	7.1 ± 0.7 (8)

Data plotted as Figure 12, overleaf. Number of animals given in parentheses.

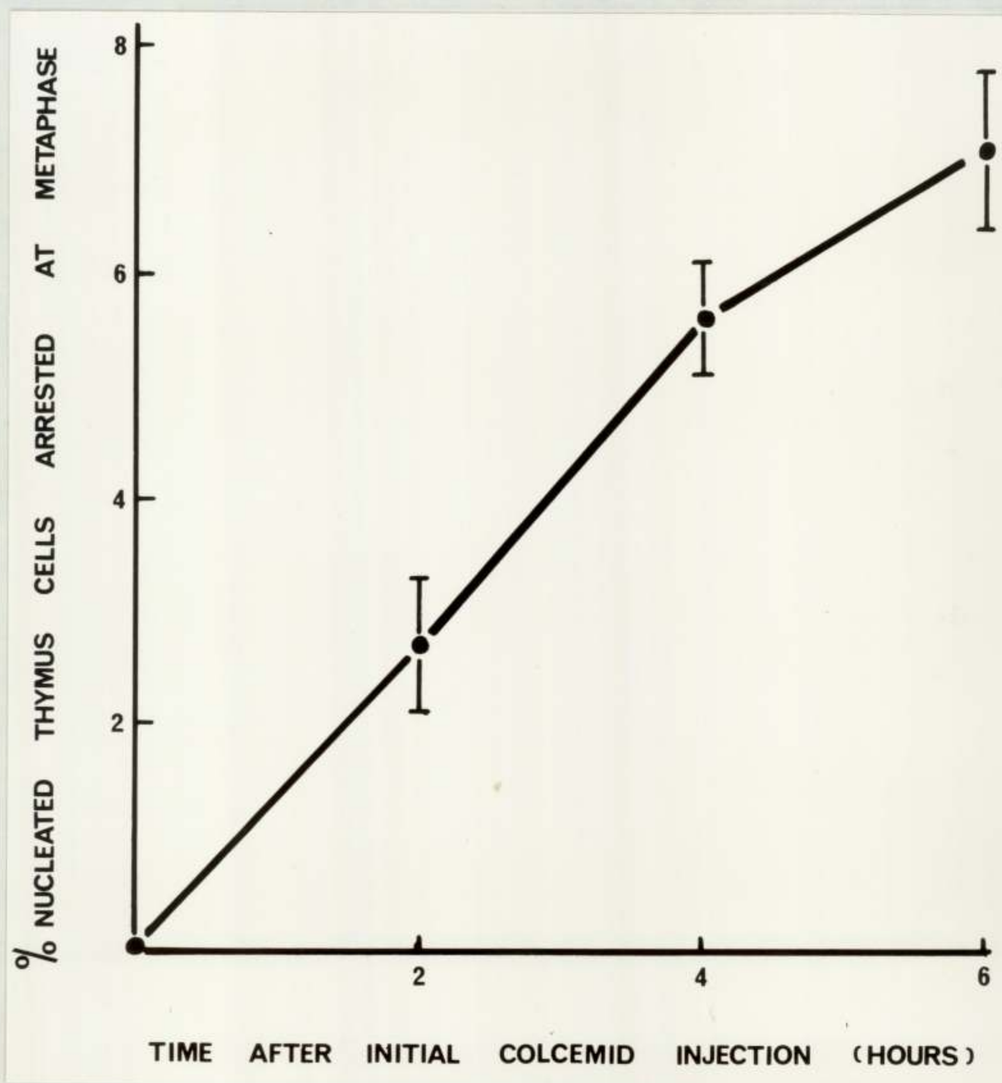


Figure 12: Linear progression of thymus cells into metaphase after Colcemid treatment *in vivo*. Colcemid (0.2 mg/100g body weight) injected at 0 and 3h. Each point and bar represents the mean ± s.e.m. derived from 4 - 14 animals.

Although there are possible limitations of the mitotic index method as a measurement of mitotic activity in tissues (see above), the method also possesses two distinct advantages over others:
(i) No administration of chemicals or handling of the animals is required. Thus artefactual or stress-induced errors are precluded.
(ii) Microscopic observation of all stages of mitosis, and of cells which have just separated after telophase, in a tissue indicates that the normal progression of the cells through mitosis has not been affected, i.e. the observed changes probably have physiological significance in the experimental animal.

In most experiments parallel determinations of mitotic activity by assessment of mitotic index and by use of colchicine have routinely been performed.

6.4 Colchicine and Colcemid.

Colchicine possesses the property of arresting a great variety of cell types in their progression through mitosis at the metaphase stage (Eigsti & Dustin, 1947, 1949). It has been demonstrated *in vivo* that over a 4 or 6h period there is an essentially linear progression of bone marrow and thymus cells into mitosis and the arrested metaphase condition (Perris *et al.*, 1967). This has been confirmed (Figures 11 and 12). Thus by scoring the percentage of nucleated cells which were in colchicine-metaphase (Mangenot, 1942; Bertalanffy & Lau, 1962) it was possible to compare the effects of different treatments upon the level of mitotic activity in the tissues.

In the present studies the related alkaloid desacetyl-N-methyl colchicine ("demecolcine" or "Colcemid", Ciba Ltd.) was routinely used because of its superior stability; its effects upon bone marrow and

Table 14 overleaf.

TABLE 14

	Plasma concentrations(mg/100ml)4h after colcemid(A)			Plasma concentrations(mg/100ml)6h after colcemid(B)		
	Saline treated	Colcemid treated	p	Saline treated	Colcemid treated	p
Total calcium	9.6 $\pm$ 0.1	9.5 $\pm$ 0.1	$>$ 0.5	10.1 $\pm$ 0.1	9.6 $\pm$ 0.2	$<$ 0.05
Ionised calcium	5.20 $\pm$ 0.05	5.25 $\pm$ 0.15	$>$ 0.5	–		
Phosphate	5.85 $\pm$ 0.10	5.65 $\pm$ 0.10	$>$ 0.2	–		
Total magnesium	1.8 $\pm$ 0.05	1.9 $\pm$ 0.05	$>$ 0.05	–		

Table 14: Effect of colcemid upon plasma contituents. In column A Colcemid (0.22 mg/100g body weight) was given i.p. in 0.9% saline at 0h. In column B Colcemid (0.2 mg/100g body weight) was given i.p. at 0 and 3h. All control animals received 1ml. 0.9% saline i.p. at the same times. Each value is the mean $\pm$ s.e.m. from 5 animals.

thymus cells in vivo are identical to those of colchicine (Rixon, 1968). A Colcemid dose of 0.05 - 0.07mg/100g body weight/hour of accumulation was found suitable to prevent cell escape from the arrested metaphase condition in the rats under study. Where anaphase or telophase figures were observed in smears the results were discarded. For a 4h accumulation period this was given as a single intraperitoneal injection in a saline vehicle; for a 6h period the dose was given as two equal injections at 0 and 3h (Perris et al., 1967).

It has been demonstrated (Rixon, 1968; Heath, Palmer & Aurbach, 1971) that 0.2mg/100g body weight of colchicine may reduce plasma calcium concentrations in young rats by inhibiting bone resorption of the ion. The effects of the normal experimental dose of Colcemid upon plasma constituents are shown in Table 14. When a single injection of 0.22mg Colcemid /100g body weight was given plasma calcium, phosphate and magnesium concentrations were unaffected 4h later, but two injections of 0.2mg/100g body weight did cause small but significant decreases in plasma calcium concentration at 6 hours. These changes could be reflected in a decrease in the number of dividing cells reaching metaphase between 4 and 6h after the start of Colcemid treatment, since it is well established that the calcium ion influences mitotic activity in bone marrow and thymus (General Introduction, Section 5). Thus the decreases in the gradients of the lines in Figures 11 and 12 which occur between 4 and 6h might be attributable to a lowering of the extracellular calcium concentration caused by Colcemid. However the short time involved between the onset of the hypocalcaemia and the termination of the accumulation period would suggest that any effect upon tissue mitosis would be small; probably the decrease in cells reaching metaphase over the last two hours of a 6 hour accumul-

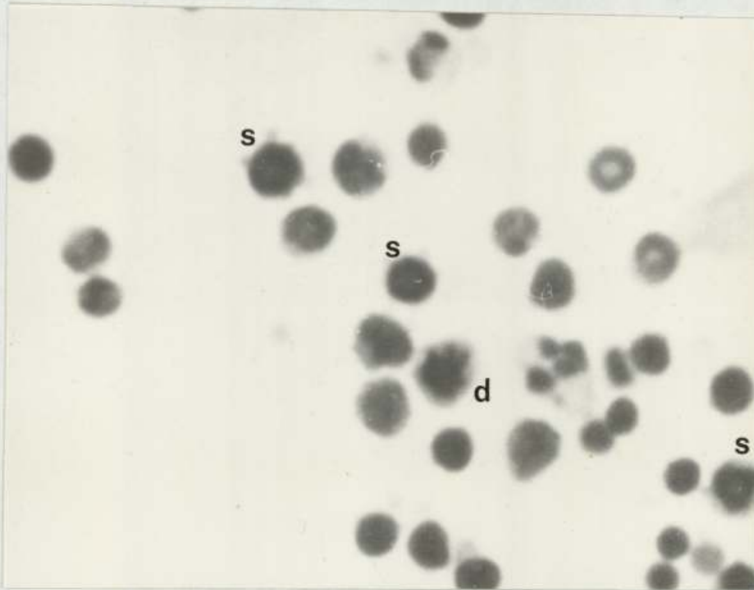
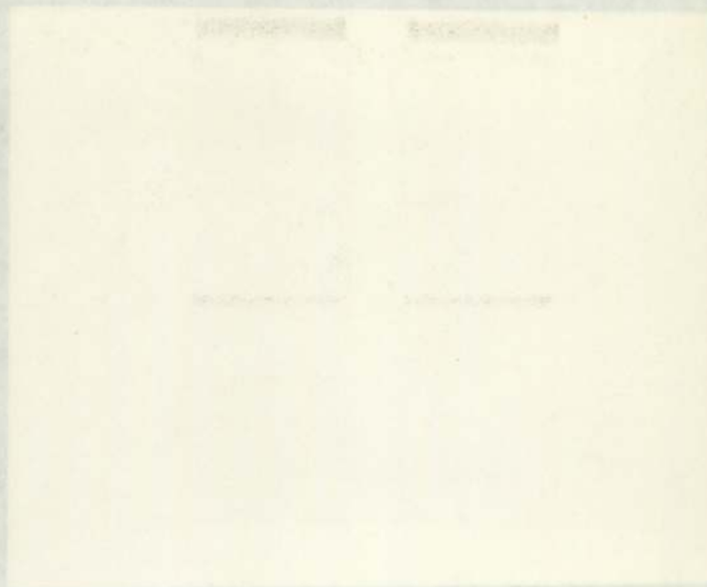


Plate 5: Colcemid metaphase figures in bone marrow cells (approx. x 1500). s = "star" metaphase; d = "distorted star".



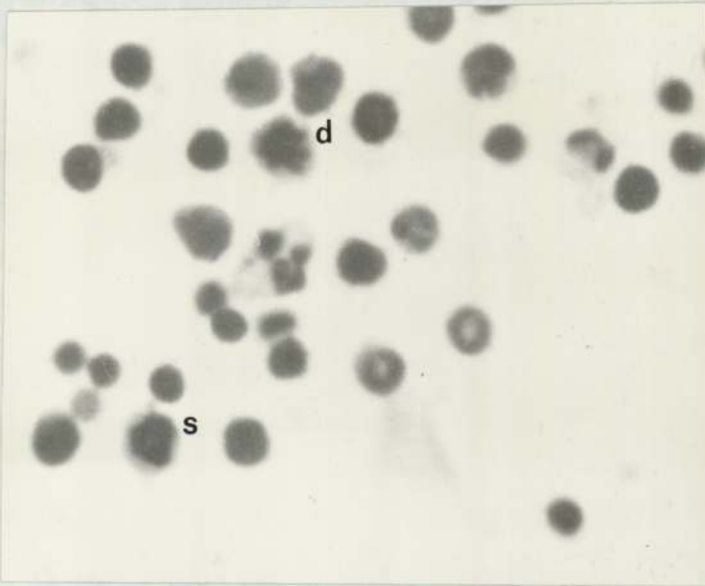


Plate 6: Colcemid metaphase figures in bone marrow cells (approx. x 1500). s = "star" metaphase; d = "distorted star".

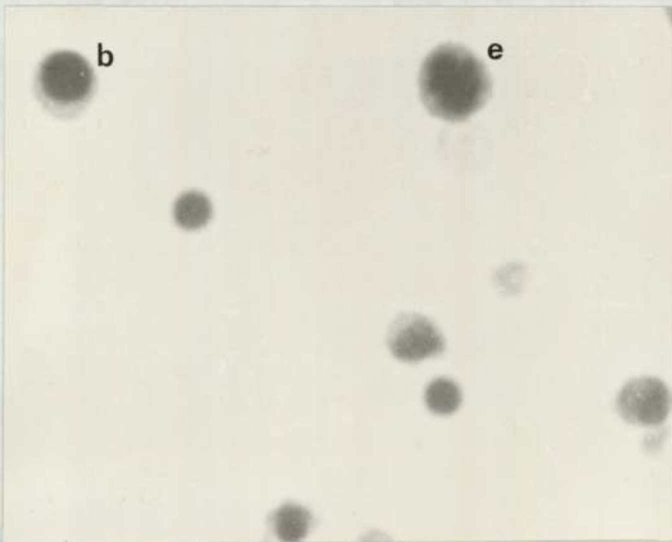


Plate 7: Colcemid metaphase figures in bone marrow cells (approx. x 1500). e = "star" metaphase beginning to escape from blockade; b = ball metaphase.

ation is a result of progressive exhaustion of cells competent to enter the S phase of the cell cycle.

Regardless of whether the hypocalcaemic action of colcemid significantly affected the flow of cells into metaphase, the use of the alkaloid over a 6h period was not invalidated as a means of comparing mitotic activity in groups of rats since the effect would have been manifest both in control and in all test groups. However, a single injection of Colcemid followed by a 4h accumulation period was used in most experiments since the complete absence of a hypocalcaemic response simplified interpretation of the data. In addition, the reduction in the number of injections administered and the reduced handling decreased the stress to which the animals were exposed.

Four major arrested-metaphase configurations, some examples of which are shown in Plates 5 - 7, have been observed in bone marrow and thymus. These have been termed star-, distorted star-, exploded- and ball-metaphases (Eigsti & Dustin, 1955).

6.5 Scoring Cell Populations.

As mentioned earlier, both bone marrow and thymus smears were scored for the percentage of nucleated cells undergoing mitosis, with or without prior treatment with Colcemid. It was therefore necessary to ensure that smears prepared from single drops drawn from much larger volumes of cell suspensions contained cell populations of approximately the same compositions as the suspensions themselves.

The distributions of the various cell sub-populations were monitored visually in a number of smears drawn from single suspensions of bone marrow and thymus cells. In addition, repeated estimations of the size distribution of cells drawn from single suspensions, using a Coulter Counter Model ZBl (see Section 8), showed a remarkable

Table 15 and Table 16 overleaf.

TABLE 15.

Cell suspension	% nucleated cells in metaphase after Colcemid	
	Mean ± s.e.m.	Range
1	14.0 ± 0.25	13.7 - 14.5
2	14.8 ± 0.3	14.4 - 15.4
3	14.2 ± 0.2	13.8 - 14.4

Table 15. Consistency of mitotic activity in smears randomly drawn from single cell suspensions from bone marrow. Four smears were prepared from each of three cell suspensions and scored for nucleated cells in Colcemid-metaphase.

TABLE 16.

Smear No.	% nucleated cells in metaphase 6h after Colcemid Treatment		
	Mean ± s.e.m. from 5 samples	Mean from first 2 samples	Range from 5 samples.
1	14.3 ± 0.35	14.5	13.2 - 15.1
2	14.2 ± 0.5	14.3	12.6 - 15.1
3	14.3 ± 0.25	14.1	13.5 - 15.0

Table 16. Consistency of estimates of mitotic activity in 1000 cell samples drawn from single smears from bone marrow. Five samples from each smear were scored for % nucleated cells in Colcemid metaphase

constancy. Thus it was unlikely than any observed variations in mitotic activity between animals could be attributed to sampling biased towards particular cell types. By preparing a series of slides from single suspensions of bone marrow cells derived from Colcemid-treated animals it was possible to demonstrate that the sampling procedure yielded smears which were also representative of the mitotic activity of the cell suspensions as a whole, and therefore the test animals, from which they were drawn (Table 15).

Representative samples of 1500 - 2500 nucleated cells per slide were counted (where Colcemid had been used). This encompassed approximately one hundred different fields. Smears stained and fixed as previously described (Section 6.2) were viewed with a Leitz binocular light microscope, using x 1300 magnification with oil immersion. It was necessary to determine whether this randomly drawn sample provided a close approximation of the level of mitotic activity represented in the smear. A number of separate 1000 cell samples drawn from single smears showed close coincidence in the percentage of nucleated cells scored at metaphase (Table 16). In addition, the mean of the first two estimations differed by only some 1 - 2% from the mean score from much larger numbers of samples (see Table 16). Thus the following protocol was usually observed: two separate 1000 cell samples were scored, often by two observers; if the two values differed by more than 8% of the mean a further 1000 cells were counted.

Normal values for mitotic index in both bone marrow and thymus are approximately 10% of the values obtained after Colcemid treatment for the percentage of cells which are trapped at metaphase in 6 hours. Thus the potential magnitude of subjective errors is greater when scoring mitotic indices than Colcemid-metaphases. The sample size for

mitotic index determinations was therefore usually taken to be 4 - 5000 cells per smear counted in two equal portions, often by two observers.

Subjective errors, e.g. mistaken indentification of mitotic figures or scoring thereof, were minimised in three ways:

(i) two separate observers counted equal samples from each slide;

(ii) the labelling of slides was often obscured so that observers were unaware of the origin of the smears; and

(iii) figures in which positive identification was difficult (which occurred infrequently) were rationalised by considering alternate ones to be positive.

The above criteria were adopted to ensure that parallel determinations of mitotic index and Colcemid-metaphase accumulation provided an accurate reflection of mitotic activity in bone marrow and thymus.

Section 7. In Vitro Culture of Thymus and Bone Marrow Cells.

7.1 General Considerations.

Since mitotic activity in bone marrow and thymus may be affected by a number of hormones in vivo (Dougherty, 1952; Santisteban & Dougherty, 1954; Lundin, 1958; Gordon, 1959; Shrewsbury & Reinhardt, 1959; Santisteban, 1960a, b; Perris et al., 1967; Perris et al., 1970; Perris & Whitfield, 1971; Results, Chapters 2 and 3), it was necessary to use in vitro culture systems to :

(i) determine whether the actions of particular agents upon mitotic activity were direct or mediated via some other physiological system; and

(ii) attempt to elucidate the mechanisms by which mitotic agents which acted directly upon cells exerted their effects.

Culture systems can at best only poorly approximate the in vivo microenvironment from which the cells have been removed. Thus any in vitro effects which are not reproducible in vivo must be considered to be of doubtful physiological significance. In the present studies, in vitro work was always inspired by previous in vivo observations and was therefore considered to be a useful supplemental tool for the purposes described above.

7.2 Thymocyte Culture.

Suspensions of cells prepared from thymus contain approximately 80 - 85% small, mitotically inert lymphocytes and 15 - 20% medium or large mitotically active lymphocytes (see General Introduction for detailed review). The latter group have a cell cycle time of only 6-8 hours (Metcalf, 1966a) which makes them suitable for short term culture. As described in the General Introduction (Section 5) sus-

Table 17 overleaf.

TABLE 17.

Compound	Concentration (mM)
Glucose	5.5
KCl	5.0
$MgSO_4.7H_2O$	1.0
$CaCl_2.2H_2O$	0.6
NaCl	120.0
$Na_2HPO_4.12H_2O$	5.0
"Tris" buffer	5.0
pH	adjusted to 7.2 with HCl

Table 17. Composition of the balanced glucose salts medium for thymocyte culture. "Tris" buffer is tris (hydroxymethyl) aminomethane buffer.

pension cultures of thymocytes have been widely used to elucidate the mechanisms of action of number of mitogenic ions and hormones.

Thymocytes were cultured in a balanced glucose-salts (BGS) medium (Whitfield, Brohée and Youdale, 1964), the composition of which is given in Table 17. Thymuses were removed from male Wistar rats weighing approximately 200g, washed and then minced in the culture medium. After filtration through moist cheesecloth the resultant thymocyte suspension was diluted with BGS to give a culture containing approximately 4×10^7 cells/ml. Since the mitotic activity of thymus cells _in vitro_ is low, Colcemid was added to the culture to give a concentration of 0.062mM; thus cells proceeding through mitosis were arrested at metaphase. The aliquots of the suspension were incubated for 6h in tubes sealed with non-toxic rubber bungs while being rotated about their long axis at 40r.p.m. at 37°C. Samples of the suspension were removed and smears prepared as previously described (Section 6.2). The percentage of nucleated cells present which had been arrested at metaphase were scored as before (Section 6.5).

Under these culture conditions there was an approximately linear accumulation of cells at metaphase with time (Figure 13; Whitfield _et al._, 1969a). There was a small (approximately 4%) reduction in total cell numbers in suspensions over the total 6h period (Morgan, 1973). The concentration of calcium and/or hormones in the suspension culture was varied in different experiments.

The technique proved satisfactory for _in vitro_ investigations such as those described in Results Chapter 6.

Culture time	% nucleated cells in metaphase
1	0.7 ± 0.1
2	1.1 ± 0.2
3	2.4 ± 0.2
4	3.3 ± 0.2
6	4.2 ± 0.3

Data plotted as Figure 13, overleaf.

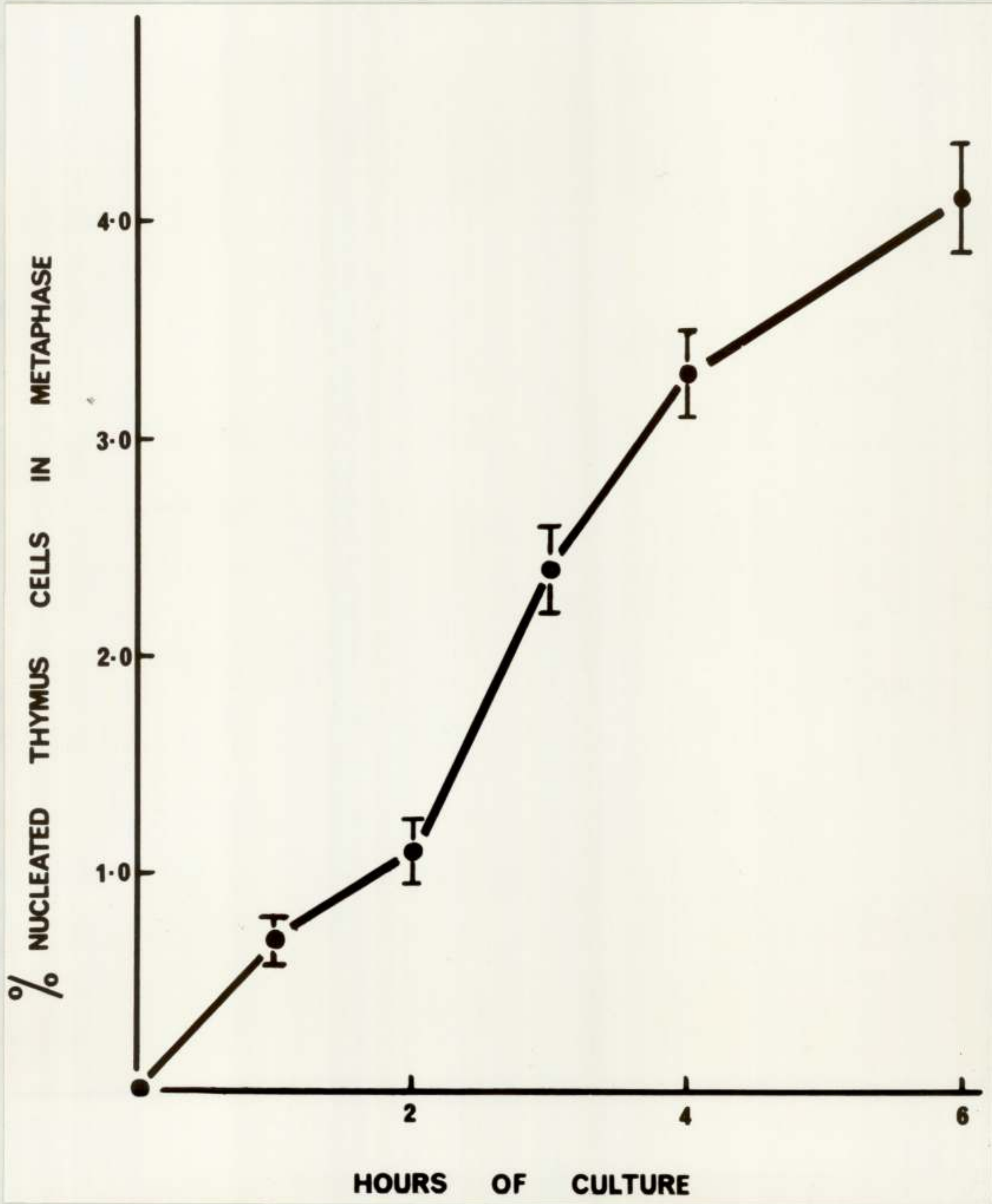


Figure 13: Linear progression of thymus cells into metaphase in the presence of Colcemid in vitro. See Section 7.2 for description of culture technique. Each point and bar represents the mean ± s.e.m. derived from 4 cultures.

7.3 Bone Marrow Culture - Description of Procedure.

After extensive preliminary experiments a modified version of the culture system of Morton (1967) and Morton & Isaacs (1967) was adopted for cultivation of bone marrow cells *in vitro*. This system is described in brief in this section with special emphasis upon the modifications which have been made to Morton's system. The rationales for the various facets of the system are discussed in Section 7.4.

In general, suspensions of rat bone marrow cells were cultured in homologous serum for varying periods of time at 37°C in a humidified atmosphere of 30% CO_2/70% air.

Homologous serum was prepared from donor blood of healthy male rats. Blood was allowed to clot for 15 minutes at room temperature (18 - 23°C) and then centrifuged at 500xg for 10 minutes. Serum was drawn off and inactivated by heat treatment (30 minutes at 56°C) in sealed containers. Inactivation averted precipitation of protein in the cultures. Serum was stored overnight at -4°C and, after thawing, was passed through 0.4μ pore size Millipore filters to exclude whole cells and large debris.

Suspensions of bone marrow cells were prepared from young (90 - 110g) male Wistar rats. Under light ether anaesthesia both femurs were removed from each rat, scraped clean and swabbed with alcohol. The epiphyses were then removed and the marrow plugs from 8 femurs extruded into 5ml homologous serum. After thorough dispersal by gentle pipetting 0.2ml aliquots of this suspension were dispersed in 1.3ml 90 - 100% homologous rat serum, containing 50 units/ml of both penicillin and streptomycin, in 30mm vented, disposable plastic petri dishes (Sterilin Products). This gave a final cell count of 4 - 9

x 10^6 cells/ml culture, of which 2.5 - 6 x 10^6 cells/ml were nucleated. Cell counts were made with a coulter counter Model ZB1 (see Section 8), with lower threshold setting 3.5 and upper setting 110.

The cultures were incubated in sealed jars in an atmosphere of 30% CO_2/70% air (Morton, 1967) for varying lengths of time, at 37°C. The atmosphere was humidified to prevent culture dessication. The cultures were not shaken during the incubation period. At the end of the culture period the cells (which did not adhere to the petri dish) were gently dispersed throughout the serum and transferred to test tubes. The tubes were centrifuged at 150xg for 30 seconds. The supernatant was discarded and the cell pellet re-suspended in 0.1ml of the culture medium. This final suspension was mixed with a single drop of calf-serum on a slide and smeared, dried and stained as previously described (section 6.2). Smears were scored for the percentage of nucleated cells present in metaphase where Colcemid was present in the culture (see below). In some experiments terminal cell counts were made to check culture viability (see Section 7.4)

Hormones, ions or Colcemid were added to the serum prior to the introduction of the bone marrow suspension. Where possible hormones were dissolved directly in the serum but in some cases they were added in small (10µl) volumes of 0.9% saline. The ionised calcium concentration of the serum was adjusted (see Section 7.4) by addition of $CaCl_2$ in 0.9% saline.

7.4 Bone Marrow Culture - Discussion of Procedure.

The following facets of the culture system require further elaboration:

(i) Cell viability and the use of Colcemid.

(ii) Serum concentration.

(iii) Incubation temperature.

Culture time (h)	% Nucleated cells in metaphase
2	1.0 ± 0.1
4	2.3 ± 0.2
6	3.1 ± 0.2

Data plotted as Figure 14, overleaf.

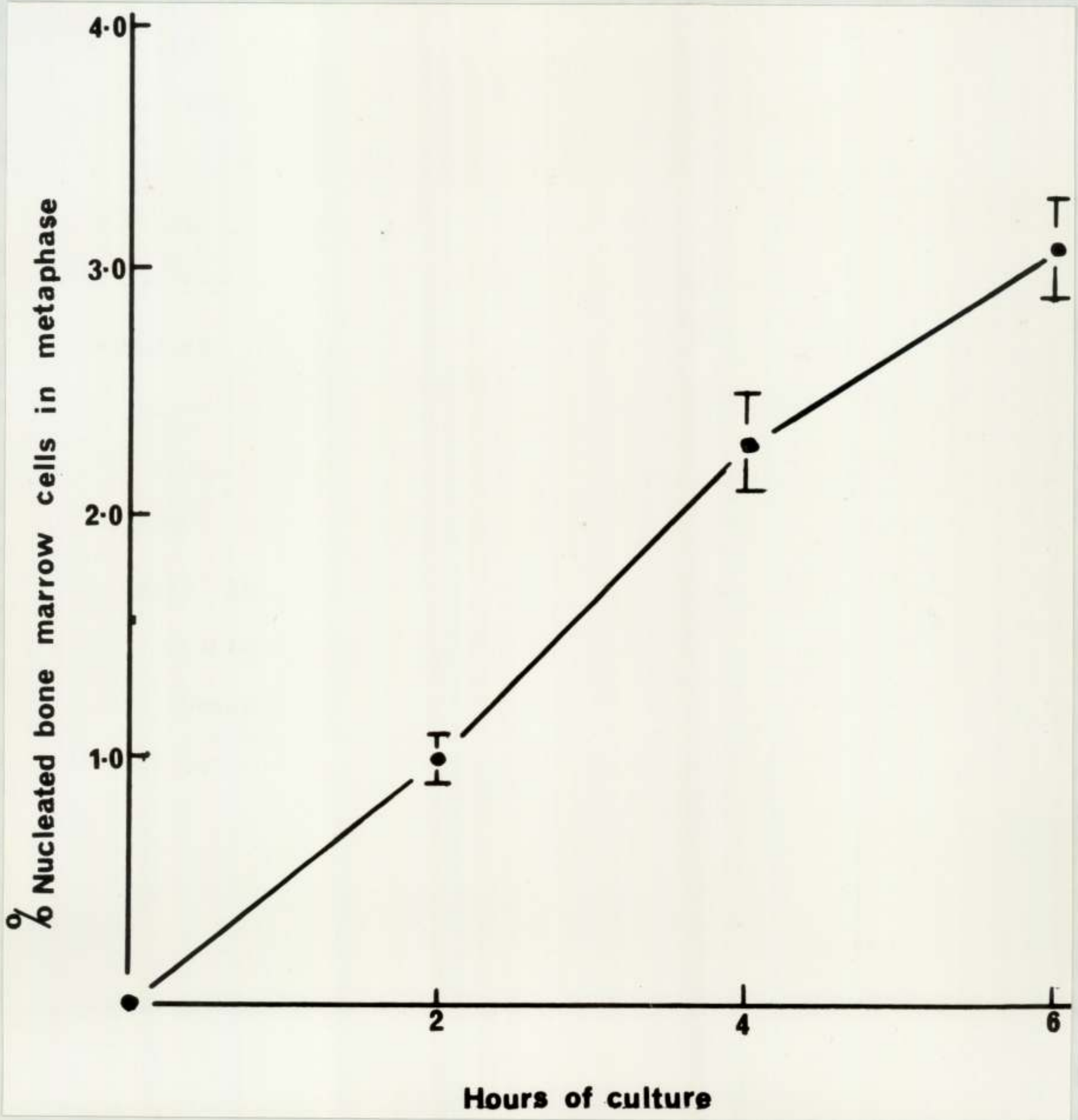


Figure 14: Linear progression of bone marrow cells into metaphase in the presence of Colcemid in vitro. See Section 7.3 for description of culture technique. Each point and bar represents the mean ± s.e.m. derived from 4 cultures.

(iv) CO_2 saturation.

(v) Adjustment of ionised calcium concentration.

(vi) Lack of mechanical disturbance.

(vii) Terminal pelleting procedure.

The use of Colcemid in short-term (4 - 6h) cultures obviously precludes an increase in cell numbers in the culture since the alkaloid arrests dividing cells at metaphase (Section 6.4). Indeed, over a 6h period there was generally a small (approximately 10%) decline in cell numbers. This decline might have been due to cell death or to lack of recovery of cells from the incubation dishes. There was, however, no noticeable adhesion to the dish (see below) and the decrease was presumably, therefore, a consequence of cell death. Preliminary experiments (values not tabulated) had indicated that approximately half of the reduction in cell numbers was within the non-nucleated fraction. Ineffective erythropoiesis (i.e. divisions of erythroid precursors resulting in cell death) is a common phenomenon <u>in vivo</u> (Stohlman, 1962) and is therefore likely to occur in the <u>in vitro</u> situation also. These considerations led to the assumption that the observed small reduction in cell numbers did not invalidate the technique. A preliminary series of cultures was prepared without the presence of Colcemid in the incubation medium. Microscopic examination revealed that in smears from cultures maintained in a variety of ionised calcium concentrations cells could be observed in all stages of mitosis. Newly divided cells were also observable. These observations indicated that bone marrow cells in the culture were capable of proceeding through mitosis in a manner morphologically indistinguishable from that seen in the whole animal. This was a further indication that the culture technique was suitable for the study of the action of mitogenic agents upon bone marrow cells. A linear progression of cells into the arrested metaphase condition was established

(Figure 14). Cell morphology was satisfactory in normal circumstances.

High concentrations of homologous serum have proved to be the most suitable media for the maintenance of normal erythropoiesis and marrow cell proliferation in vitro (Morton & Isaacs, 1967). This is presumably because serum contains the wide range of substances essential for cell growth. The availability of iron and ionised calcium (see below) are probably of particular importance. Serum from female donors was not used since it has been demonstrated that oestrogen inhibits calcium-induced mitogenesis in rat thymocytes and bone marrow cells in vivo and in the former in vitro (Morgan & Perris, 1974; Smith et al., 1974) and might well have exhibited this property in the bone marrow cultures.

Several authors (Erslev & Hughes, 1960; Smith & McKinley, 1965; Morton & Isaacs, 1967) have stressed the beneficial effects of incubation at 32°C in long term bone marrow cultures ($>$ 5 days). It was considered that in the short term cultures essayed in these studies the ambient temperature should be maintained at normal physiological temperature (37°C). Note that higher temperatures tend to increase the binding of free calcium to protein in solution (Hansen & Theodorsen, 1971; Section 5.3). The beneficial effect of low temperatures might therefore result in part from the (admittedly small) elevation of the levels of free calcium ions in the medium as compared to those in serum maintained at 37°C. The beneficial effect of elevated calcium ions upon erythropoiesis in vitro is well established (Morton, 1968; Morton et al., 1968; Results Chapter 5). Maintenance of bone marrow cultures in an atmosphere rich in CO_2 has been demonstrated to improve the maintenance of erythropoiesis in this system (Morton, 1967). This property is independent of the oxygen content of the incubation atmosphere (Morton, 1967). Carbon dioxide saturation of solutions decreases

pH (see Section 5.3). However, the stimulatory effect of CO_2 on erthropoiesis has been shown to be dependent also upon the concentration of serum present in the cultures (Morton, 1967). Thus although the pH in a range of cultures containing 5 - 100% serum was constant at pH 6 - 6.4, optimal reticulocyte production was obtained only in the more highly concentrated sera. Morton has postulated that this interaction could be a result of:

(i) activation of an inactive serum component (possibly EPO) which could then stimulate erythropoiesis; or

(ii) the stimulation of the entry of the calcium ion into primitive bone marrow cells.

It is unlikely that serum from rats which had been subjected to no previous erythropoietic stimuli would contain large quantities of EPO. However, it is possible that small quantities of EPO or other erythropoietic stimulants in serum (e.g. iron) might be sufficient to affect erythropoiesis in the culture system. It seems probable, however, that the extracellular calcium ion concentration in this system is more likely to be the crucial factor (Morton, 1968; Morton <u>et al.</u>, 1968; Results, Chapter 5). Carbon dioxide saturation of the medium reduces pH (see above; also Section 5.3) which consequently elevates the proportion of calcium in serum which is available in the ionised form (Section 5.3). Diluted serum solutions in which the total calcium concentration is kept constant will obviously contain a high proportion of ionised calcium. However the beneficial effect of transferring cultures from low to high CO_2 atmosphere (Morton, 1967) is very probably a consequence of the elevation of the ionised calcium concentration in the medium. Elevated extracellular ionised calcium concentration is then envisaged as stimulating mitosis as it does in a number of situations (General Introduction, Section 5; Results, Chapter 5).

Table 18 overleaf.

Sample no.	a (g/100ml)	b (mg/100ml)	c (mg/100ml)	$\frac{c \times a}{b}$ (= k)
1	7.6	10.4	5.15	3.74
2	7.0	10.5	5.55	3.72
3	8.9	10.3	4.40	3.80
4	7.5	10.0	4.65	3.68
5	8.0	10.2	4.75	3.70
6	7.4	10.1	5.05	3.69
7	7.2	10.0	5.10	3.67
8	8.4	10.3	4.60	3.77
9	7.4	10.3	5.20	3.76
10	7.3	10.2	5.00	3.82
11	7.5	10.1	5.10	3.79
Mean	7.7	10.2	4.95	3.74
Range	7.0 - 8.9	10.0 - 10.5	4.40 - 5.55	3.67 - 3.82

Table 18: Empirical derivation of the relationship between the concentrations of total (b) and ionised (c) calcium and protein (a) in serum at constant temperature in a constant CO_2 atmosphere. For full discussion see text.. Relationship is given by:

$$c \times \frac{a}{b} = 3.74 \text{ (range 3.67 - 3.82)}$$

The above considerations, and the other observations which have demonstrated the crucial role of the calcium ion in the initiation of DNA synthesis and division (Perris, 1971; Whitfield et al., 1973c; General Introduction, Section 5), made the control of the ionised calcium concentration in the medium of prime importance. It was necessary to devise a system whereby all cultures could be incubated in similar calcium environments. Sera obtained from different animals obviously contain different concentrations of total calcium and calcium-binding protein. It was therefore necessary to investigate the numerical relationship between the concentrations of total calcium, ionised calcium and protein in the serum under culture conditions. Note that if the temperature and pH of the medium are constant these two factors can be excluded from consideration in the relationship. Temperature was always 37°C (see above and Section 7.3) and the pH of solutions in a constant CO_2 atmosphere is the same (cf. Morton, 1967; Section 5.3).

The concentrations of the three serum constituents were measured (see Sections 5.2, 5.3 and 5.6) in samples drawn from serum incubated at 37°C in 30% CO_2/70% air for various periods up to 6h. Serum samples were covered with paraffin oil immediately after sampling to preserve CO_2 saturation (see Sections 5.1 and 5.3). Ionised calcium concentrations were measured at 21°C and corrected for the small error introduced by this reduction in temperature (see Section 5.3). The concentrations of the three serum constituents were essentially unchanged over the 6h incubation period in the humidified atmosphere. From the values thus obtained (Table 18) it was possible to establish the following empirical relationship:

$$c \times \frac{a}{b} = 3.74 \text{ (range } 3.67 - 3.83) \quad (4)$$

Table 19 overleaf.

a (g/100ml)	b (mg/100ml)			c (mg/100ml)		
	Initial	Final (Predicted)	Final (Observed)	Initial	Final (Predicted)	Final (Observed)
7.8	10.7	11.7	11.7	5.05	5.50 - 5.75	5.60
7.5	10.3	11.3	11.3	5.10	5.55 - 5.80	5.60
7.9	10.4	12.4	12.3	4.95	5.70 - 5.95	5.90
8.6	9.9	11.9	11.9	4.35	5.10 - 5.35	5.40
8.3	10.0	13.0	12.8	4.60	5.70 - 5.95	5.85
8.1	10.1	13.1	13.0	4.75	5.90 - 6.15	5.90

Table 19: Prediction of serum ionised calcium concentration. Serum total calcium concentration (b) was adjusted by addition of $CaCl_2$ while serum protein concentration (a) remained constant. Ionised calcium concentration (c) was measured in the serum and compared with the range predicted by:

$$c \times \frac{a}{b} = 3.74 \text{ (range 3.67 - 3.82)}$$

See text for full discussion of the derivation and application of the equation. All values of a, b, and c are for serum at 37°C in an atmosphere of 30% CO_2, 70% air.

Where a = concentration of protein (g/100ml)

b = concentration of total calcium (mg/100ml)

c = concentration of ionised calcium (mg/100ml).

Therefore if the concentrations of total calcium and serum protein in a serum sample were measured it was possible to adjust the concentration of ionised calcium to the required value by addition of $CaCl_2$, for:

$$1.5 \times 10^{-5}(b_1 + b_2) = \frac{a \times c}{3.74} \qquad (5)$$

Where $b_1(1.5 \times 10^{-5})$ = g $CaCl_2$ present in 1.5ml culture.

$b_2(1.5 \times 10^{-5})$ = g $CaCl_2$ required to adjust Ca^{++} in 1.5ml culture.

The validity of the relationship was established by adding predicted amounts of $CaCl_2$ to aliquots of serum and measuring the ionised calcium concentration after CO_2 saturation. The values obtained were observed to fall within the range predicted by the limits of the constant established in equation (4) (Table 19).

Because the endogenous total calcium content of serum is high it was not possible to adjust the ionised calcium concentration to 0.6mM (2.4mg/100ml), which is the basic "low calcium" medium used for thymocyte cultures (section 7.2; Table 17), without dilution, which was undesirable (see above). Therefore the basic ionised calcium concentration was set at 1.2mM (4.8mg/100ml) which is close to the concentration in plasma in the rat (Results, Chapters 1, 3 and 4; Appendix I).

In some preliminary cultures slow shaking of the incubation jar was employed to prevent cell adhesion to the petri dish. This was later found to be unnecessary.

To produce a cell density suitable for scoring for mitotic figures in the smears prepared from the cultures, cells were pelleted and resuspended in a small volume as described above (Section 7.3). Exam-

ination of the discarded supernatant showed no nucleated cells and cell debris (which would indicate mechanical disruption) was not increased in the smears.

In summary, the above observations on cell viability and morphology, together with those of Morton (Morton, 1967; Morton & Isaacs, 1967; Morton, 1968; Morton et al., 1968), were considered as good evidence that the system was suitable for the study of bone marrow mitotic activity in vitro.

Lower threshold setting	Red cell count (x 10^{-3})
2	57.4
3	56.3
4	55.8
5	47.1
6	35.9
8	13.9
10	6.6
12	2.5
14	1.3
16	0.6
18	0.3

Data plotted as Figure 15, overleaf.

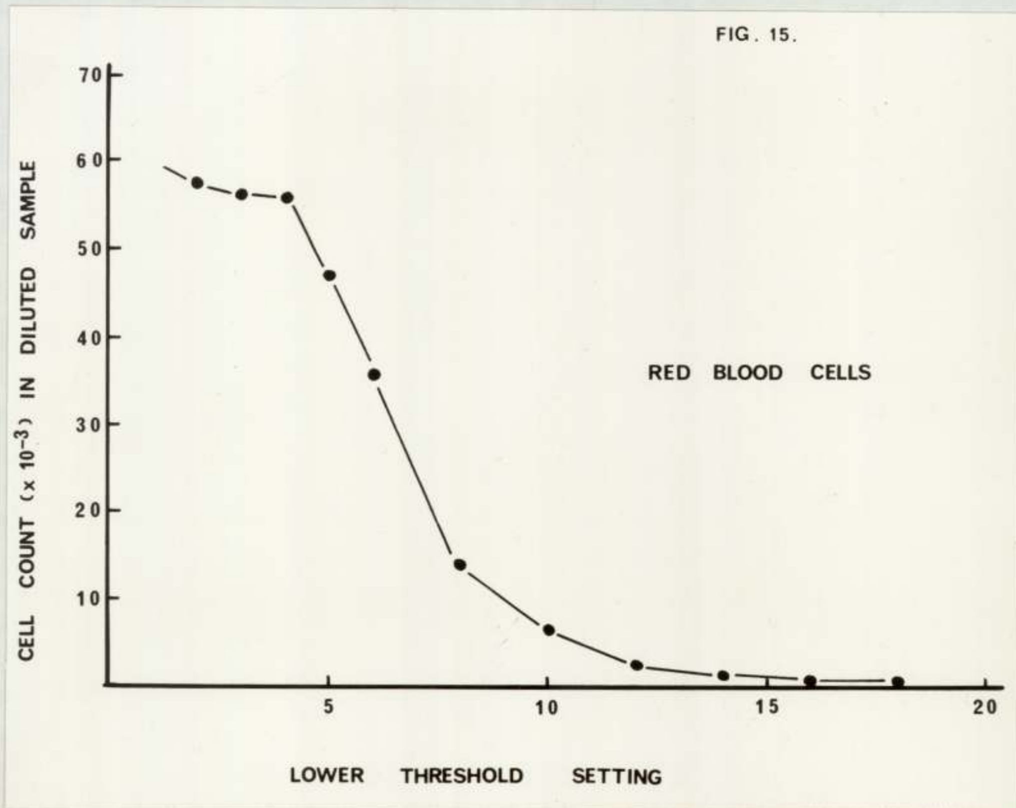


Figure 15: Determination of optimal lower threshold setting for red blood cell counts with the Coulter Counter. Each point is the mean of 2 determinations.

Lower threshold setting	White cell count ($x10^{-3}$)
2	12.4
3	11.0
4	10.6
5	9.6
6	7.0
8	3.4
10	1.0
12	0.4
14	0.1

Data plotted as Figure 16, overleaf.

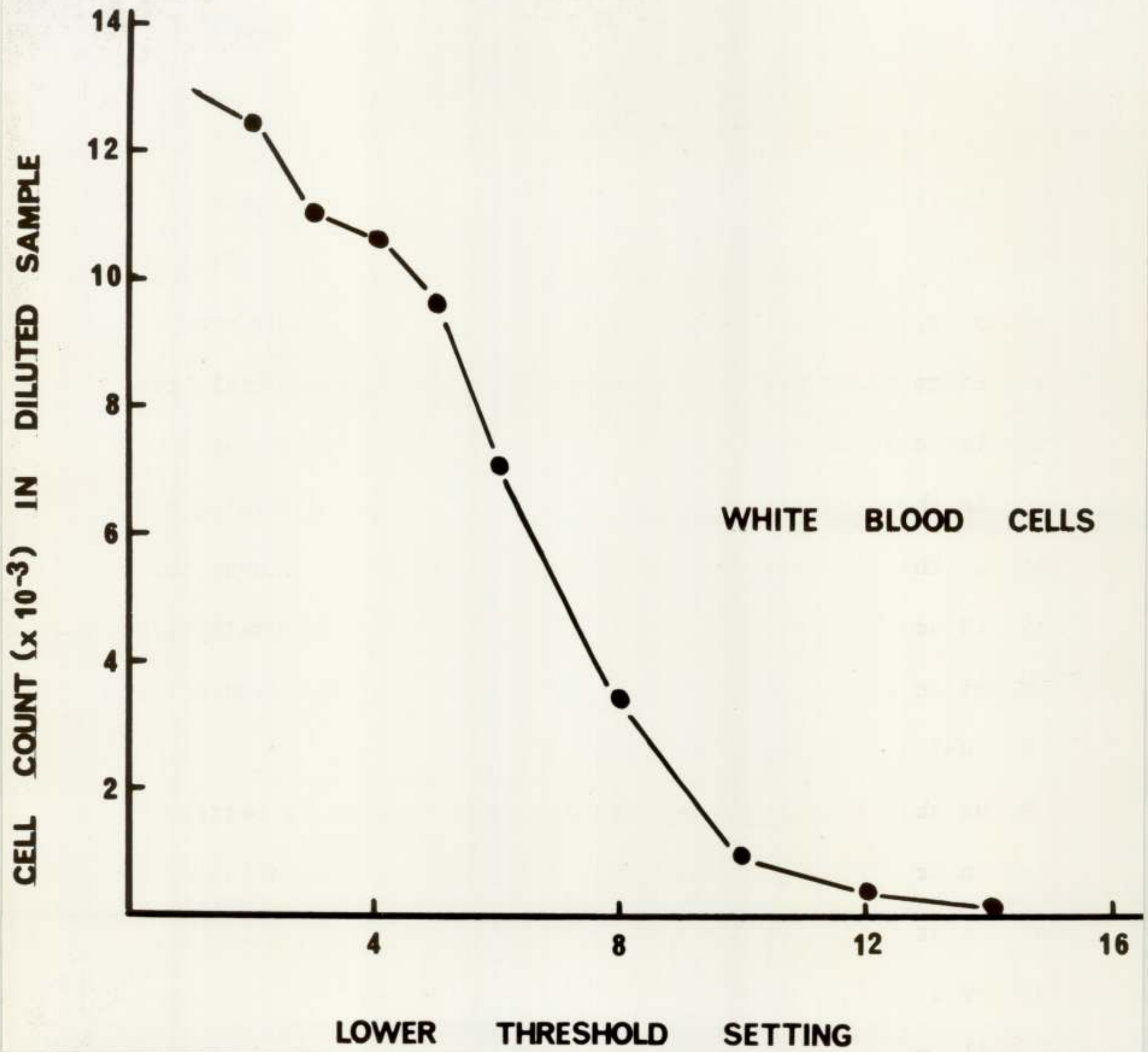


Figure 16: Determination of optimal lower threshold setting for white blood cell counts with the Coulter Counter. Each point is the mean of 2 determinations.

Section 8. Cell Counting.

An electronic cell counter — Coulter Counter Model ZB1 (Coulter Electronics) — was used for counting absolute numbers of white and red blood cells and bone marrow and thymus cells in suspension; the size distribution of cell populations was also measured.

The cell counter draws a fixed volume of a cell suspension through a small aperture flanked by two electrodes. As a cell passes through the aperture it displaces an equal volume of conducting medium which causes an impedance shift between the electrodes, thereby producing a short voltage pulse with a magnitude proportional to particle size. To minimise coincidence, that is the simultaneous passage of two or more cells through the aperture, cell suspensions are diluted before counting. The manufacturer provides a chart which estimates the level of coincidence in these dilute suspensions, based upon statistical considerations. An accuracy of ± 1% is claimed by the manufacturer; this has not been investigated.

Adjustable threshold controls allowed counts to be restricted to particular size ranges. Typical curves for determination of optimal lower threshold settings are shown in Figures 15 and 16. From these curves the settings were derived as 3 and 3.5 for red and white blood cells respectively. For white cell measurements, red cells were first destroyed with the lysing agent "Zaponin" (Coulter Electronics); this substance was suitable for destroying other non-nucleated cells e.g. reticulocytes in bone marrow suspensions. Background counts of foreign particles in suspension were usually negligible in comparison to cell counts, with or without the prior use of Zaponin.

The repeatability and reproducibility of the method were good (Tables 20 and 21). A series of repeat measurements from single

Table 20 overleaf.

Sample No.	Red Blood Cells ($\times 10^6/mm^3$)		White Blood Cells ($\times 10^3/mm^3$)	
	Mean ± s.e.m.	Range	Mean ± s.e.m.	Range
1	4.86 ± 0.01	4.82 - 4.91	8.57 ± 0.06	8.40 - 8.81
2	5.06 ± 0.02	5.00 - 5.12	9.68 ± 0.09	9.40 - 9.88
3	4.82 ± 0.03	4.76 - 4.92	8.43 ± 0.07	8.23 - 8.62

Table 20: Repeatability of the electronic cell counter method for measuring absolute cell numbers in suspension. Means derived from 6 determinations.

Table 21 overleaf.

Sample No.	Red blood cells ($x10^6/mm^3$)		White blood cells ($x10^3/mm^3$)	
	Mean ± s.e.m.	Range	Mean ± s.e.m.	Range
1	5.10 ± 0.22	4.61-5.88	9.45 ± 0.35	8.40-10.68
2	5.25 ± 0.19	4.82-6.00	9.46 ± 0.38	8.29-10.58
3	4.98 ± 0.26	4.24-5.79	9.70 ± 0.32	8.64-10.65

Table 21: Reproducibility of the electronic cell counter method for determing absolute numbers of cells in suspension. Samples were divided into aliquots before dilution. Means derived from 4 measurements.

samples yielded an average s.e.m. which was less than 1% of the mean for both white and red blood cells (Table 20). A number of blood samples were divided prior to dilution; the absolute numbers of red and white cells in each aliquot were counted. The average s.e.m. was approximately 5% of the mean for both cell types (Table 21); thus the reproducibility of the method was satisfactory. It was observed that cell numbers in diluted suspensions began to decrease significantly 20 minutes after the dilution procedure. This was presumably due to a combination of cell lysis and settling. Thus when the apparatus was applied to the counting of absolute numbers of cells in suspensions, measurements were always made directly after suitable dilution of the original suspension.

Section 9: Summary.

A number of surgical and analytical methods have been discussed. For non-quantitative methods this discussion has taken the form of rationalised description. Quantitative methods have been investigated in depth to determine their applicability and minimise possible errors. Errors inherent in techniques (both mechanical and subjective) have been quantitatively assessed. The limits of precision for individual methods have been stated, as have the conditions in which the techniques are applicable.

RESULTS

CHAPTER 1.

Circadian rhythms in plasma calcium concentration and tissue mitosis in the male rat.

The studies reported in this Chapter have been submitted for publication (N.H.Hunt & A.D. Perris (1974), "Calcium and the control of circadian mitotic activity in rat bone marrow and thymus", J. Endocr., in press).

Introduction.

The influence of the extracellular calcium ion concentration upon mitotic activity in rat bone marrow and thymus has been thoroughly documented (Perris, 1971; Whitfield et al. 1973; General Introduction, Section 5). Furthermore it has been established that the mitotic response of these two tissues to a variety of physiological demands for increased cell proliferation is effected by hormone-mediated changes in calcium homeostasis. For example, the increase in bone marrow mitosis which follows haemorrhage, and the increased proliferation of both bone marrow and thymus during rapid growth appear to depend upon parallel changes in the concentration of ionised calcium in the plasma (Perris et al. 1968; Perris et al. 1971).

These relationships suggested that the circadian periodicity in mitosis which has been observed in a number of tissues, including bone marrow, in the rat (Cardoso & Ferreira, 1967; Clark & Korst, 1969) might be associated with plasma calcium fluctuations governed by the calcium homeostatic hormones.

The following study was therefore undertaken to:

(i) confirm that circadian changes in bone marrow mitotic activity did occur;

(ii) establish whether periodic changes in thymic mitotic activity occurred;

(iii) determine whether rhythmic fluctuations in plasma calcium existed and, if so, whether these changes paralleled those in tissue mitotic activity;

(iv) determine whether the changes in mitotic activity were dependent upon variations in plasma calcium concentration;

(v) determine the aetiology of changes in plasma calcium concentration; and

(vi) enable future studies to be planned in the light of the above observations, to preclude the possibility of circadian variations obscuring or reinforcing changes in experimental parameters.

Methods.

The general approach was to measure the plasma concentrations of various substances known to be influenced by the calcium homeostatic hormones and, simultaneously, to assess mitotic activity in the bone marrow and thymus of the rats. Data were then compared statistically to determine whether numerical relationships existed between the various parameters.

For one month prior to the experiments the animals were maintained in conditions in which external stimuli were minimised. In this way it was hoped to isolate circadian changes, which are controlled by specific cues, or Zeitgebers (Aschoff, 1954), from changes produced by non-specific factors. Thus the animal room was light for 12h (08.00 - 20.00) and dark for the remaining 12h of each day; under these conditions rats quickly conform to a circadian periodicity in activity and food and water intake (Besch, 1969, 1970). To further reinforce these rhythms animals were initially allowed access to food only during the dark period; in some later experiments food was available ad lib. Tap water was available at all times. Room temperature was maintained within the range 15-20°C; humidity was not controlled. The animals were completely undisturbed during the conditioning period, except for feeding and cleaning at 20.00 each day, since noise stimuli may cause increases in circulating corticosteriods (Barret & Stockhom, 1963) which influence production of leucocytes and thymocytes (Dougherty, 1952; Santisteban, 1960a, b). Groups of 5 rats, weighing 200-250g, were randomly selected from the large conditioned population at 4h intervals and used for the measurement of plasma constituents and mitotic activity as mentioned above.

The plasma concentrations of total and ionised calcium and inorganic phosphate are known to be influenced by the activity of the calcium homeostatic hormones PTH and CT (Hirsch, Voelkel & Munson, 1964; Rasmussen, Arnaud & Hawker, 1964; Copp, 1969a) and were therefore measured, as was the plasma total magnesium concentration. Magnesium homeostasis has some links with calcium homeostasis (MacIntyre, Boss & Troughton, 1963; Palmieri, Thompson & Eliel, 1969; Care, Bell & Bates, 1971; Nielsen, Buchanan-Lee, Mathews, Mosley & Williams, 1971) and the ionic form is a powerful stimulant of mitosis in bone marrow and thymus when injected into rats (Perris et al., 1967). Thus natural variations in plasma magnesium levels might have influenced mitotic activity in the test tissues in the present study. Blood samples were taken by cardiac puncture (General Methodology, Section 4.3) and plasma was prepared, stored and analysed as previously described (General Methodology, Section 5.1).

Suspensions of bone marrow and thymus cells were prepared, fixed, stained and scored for mitotic index as described earlier (General Methodology, Section 6.2 and 6.3).

In later experiments the percentage of nucleated cells in metaphase occurring 4h after Colcemid treatment was determined; the rationale for the use of Colcemid has been described earlier (General Methodology, Section 6.3 and 6.4). The use of Colcemid over this short 4h period did not obscure any circadian variations in the concentrations of the plasma constituents since they have been shown to be unaffected by the alkaloid within 4h of its administration (General Methodology, Section 6.4, Table 14).

To investigate the possible role of the parathyroid glands in the maintenance of circadian changes in plasma calcium concentration conditioned animals were parathyroidectomised and maintained according to the schedule previously described (General Methodology, Section 4.2). Plasma constituents and mitotic activity in bone marrow and thymus

were then measured as above.

Time of Day	Plasma Calcium Concentration (mg/100ml)	
	Total	Ionised
00.00	9.95 ± 0.1	4.65 ± 0.05
04.00	10.2 ± 0.2	4.90 ± 0.10
08.00	10.2 ± 0.2	4.65 ± 0.15
12.00	10.25 ± 0.2	4.80 ± 0.10
16.00	10.2 ± 0.1	4.75 ± 0.05
20.00	9.6 ± 0.1	4.35 ± 0.05

Data plotted as Figure 17 overleaf.

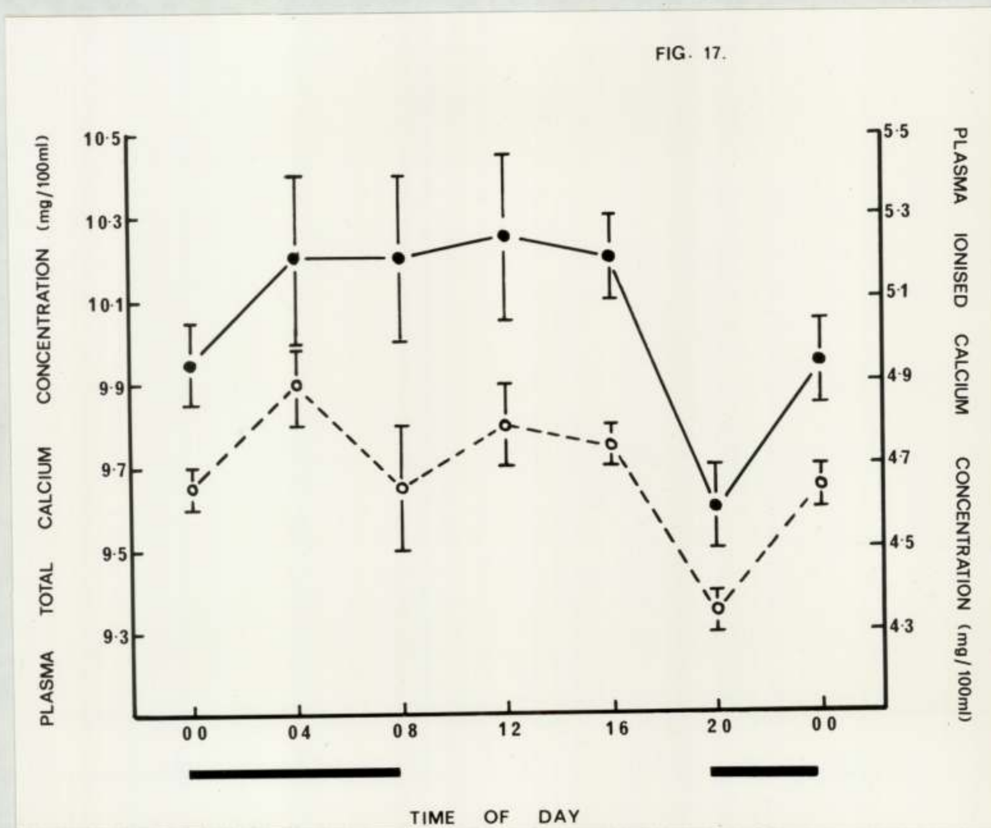


Figure 17: Mean concentrations of total and ionised calcium in plasma of rats at various times over a 24h period. The minimum concentrations of both total (solid line) and ionised (broken line) calcium were significantly different from all other values (P ranged from <0.05 to <0.01 and from <0.05 to <0.001 respectively). Points and vertical bars represent means $\pm$ s.e.m. derived from 9 - 10 animals in each case. The dark horizontal bar represents the dark period (food available).

Time of Day	Mitotic Index	
	Bone Marrow	Thymus
00.00	1.2 ± 0.1	1.0 ± 0.0
04.00	1.2 ± 0.1	0.9 ± 0.1
08.00	1.5 ± 0.1	1.2 ± 0.1
12.00	1.6 ± 0.1	1.15 ± 0.1
16.00	1.4 ± 0.1	0.9 ± 0.1
20.00	1.0 ± 0.1	0.7 ± 0.1

Data plotted as Figure 18 overleaf.

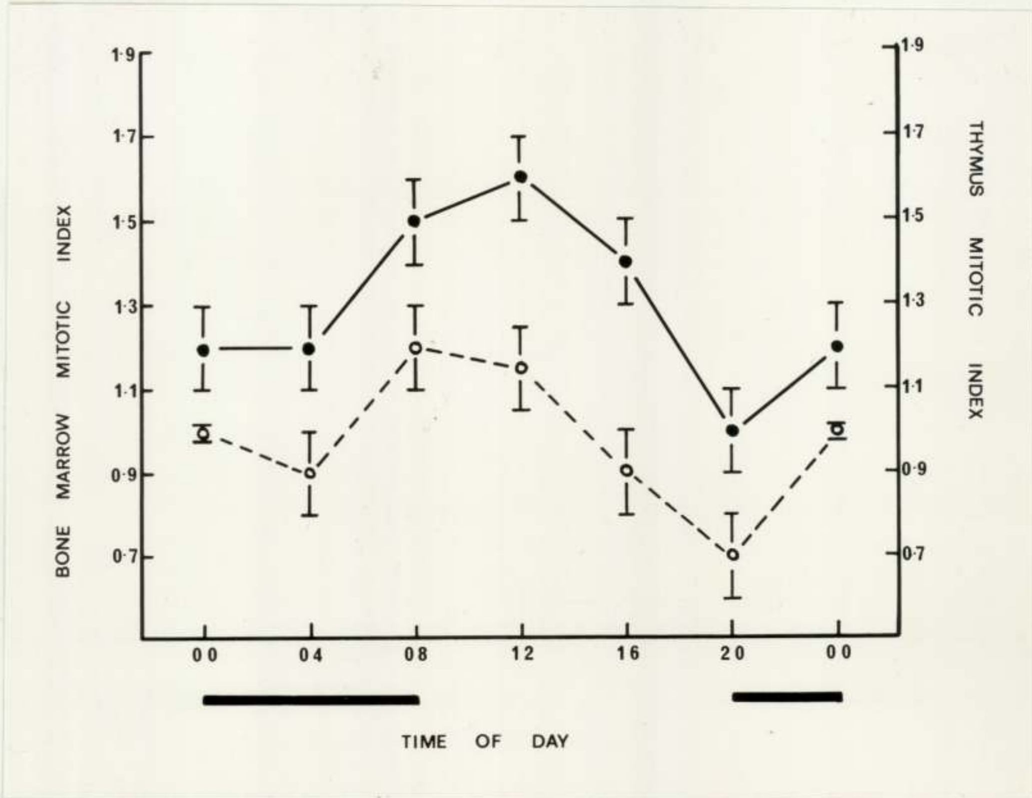


Figure 18: Mean mitotic index in bone marrow and thymus assessed at 4h intervals over a 24h period in the rat. The minima for both bone marrow (solid line) and thymus (broken line) were significantly different from all other values (P ranged from <0.05 to <0.01 and from <0.05 to <0.001 respectively. Points and vertical bars represent mean $\pm$ s.e.m. derived from 5 animals in each case. Dark horizontal bar represents the dark period (food available).

Results.

Marked changes in the plasma concentrations of both total and ionised calcium were observed in the conditioned animals during the 24h period (Figure 17). Between 04.00 and 16.00 the total calcium concentration of plasma remained fairly constant. However at 16.00 a sudden and highly significant ($P < 0.01$) fall ensued. This hypocalcaemic phase was of short duration; 4h after its onset plasma concentration had increased to a value not significantly different ($P > 0.1$) from that observed during the plateau period described above. The changes in the ionised calcium concentration of plasma over the 24h paralleled those in total calcium (Figure 17); the small additional decrease at 08.00 was not significant ($P > 0.1$). The plasma ionised : total calcium ratio was maintained within the range 0.43-0.51:1 throughout the 24h period; the two factors were closely correlated ($r = 0.86$, where $P < 0.02$). It should be noted that the onset of the sharp decreases in both total and ionised calcium concentrations occurred prior to the advent of darkness and the availability of food.

The mitotic index of both bone marrow and thymus also exhibited significant variations during the 24h period (Figure 18). In both tissues the mitotic activity was lowest at 20.00, i.e. at the time when both total and ionised calcium levels in the plasma had reached their nadirs (compare Figures 17 and 18). In view of the evidence that changes in plasma calcium concentration produce changes in mitotic activity in bone marrow and thymus (e.g. Perris et al. 1967, 1968, 1971; Rixon, 1968; Perris, Weiss & Whitfield, 1970; Perris & Whitfield 1971), the similarities in timing of the fluctuations in plasma calcium concentrations and mitotic indices in the two tissues suggested that a causal relationship might exist between the parameters.

Figure 19A/B overleaf. Data derived from Figures 17 and 18.

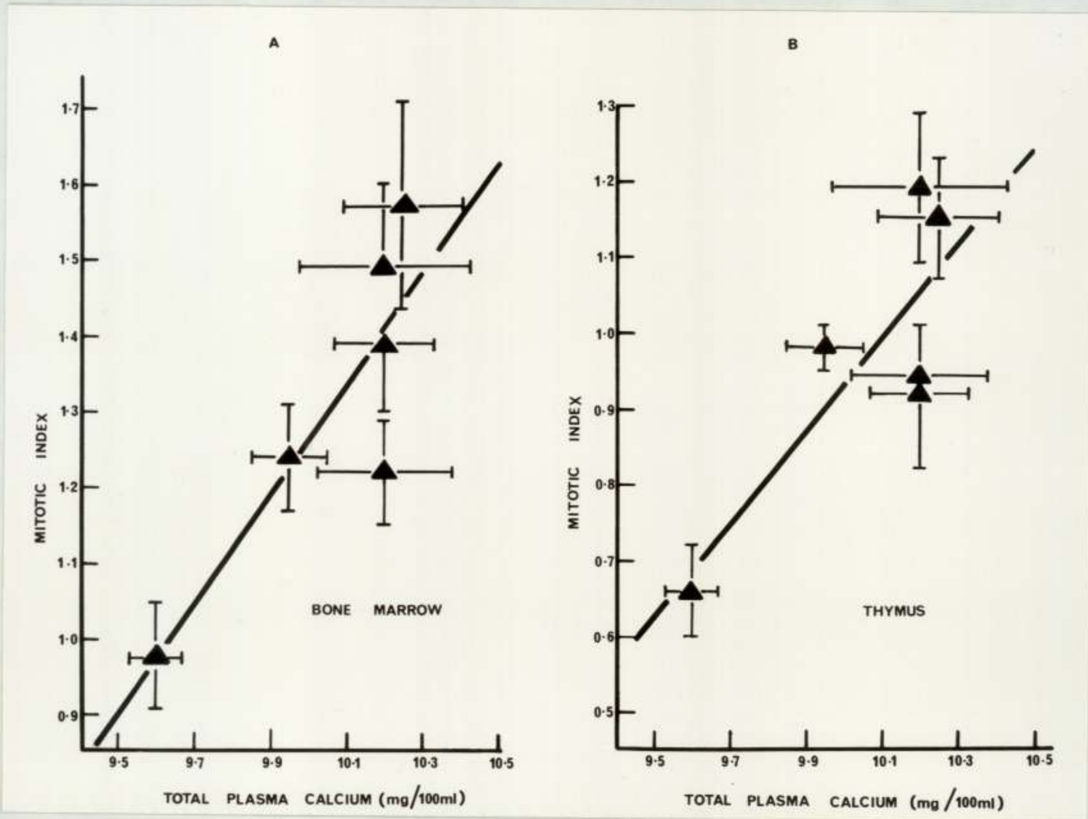


Figure 19 A/B; The relationship between the mean values of plasma total calcium concentration and (A) bone marrow or (B) thymus mitotic index at the times studied. The lines of best fit are defined by (A) $y = 0.725x - 5.98$, where $r=0.88$ and $P < 0.01$ and (B) $y = 0.61x - 5.20$, where $r=0.82$ and $P < 0.05$. Points and bars are means ± s.e.m. for both parameters from 5 - 10 animals in each case.

Time Period Ending At:	% Nucleated Cells in Metaphase 4h After Colcemid	
	Bone Marrow	Thymus
00.00	7.7 ± 0.9	4.55 ± 0.3
02.00	8.8 ± 1.0	4.3 ± 0.6
04.00	8.6 ± 0.4	4.8 ± 0.3
06.00	10.2 ± 1.3	5.5 ± 0.4
08.00	10.8 ± 0.6	6.2 ± 0.6
10.00	10.2 ± 0.7	5.7 ± 0.3
12.00	11.3 ± 0.5	4.9 ± 0.5
14.00	11.3 ± 0.9	5.2 ± 0.2
16.00	14.3 ± 0.3	6.05 ± 0.3
18.00	12.1 ± 0.6	5.65 ± 0.4
20.00	9.7 ± 0.2	5.0 ± 0.2
22.00	8.8 ± 0.7	3.8 ± 0.3

Data plotted as Figure 20 overleaf.

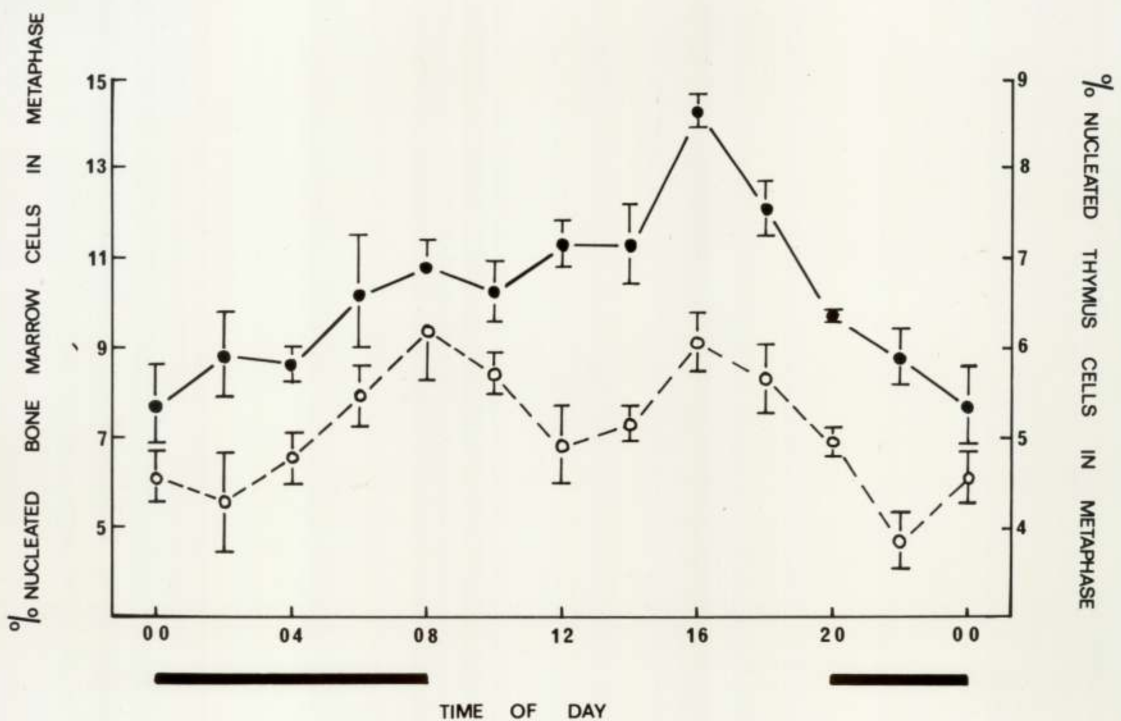


Figure 20: Mean mitotic activity of rat bone marrow and thymus assessed by Colcemid metaphase accumulation over various 4h time periods. Colcemid (dose and protocol, see General Methodology, Section 6.4) was injected 4h before death which occurred at the times shown. For both bone marrow (solid line) and thymus (broken line) the percentage of nucleated cells trapped in metaphase over the period 16.00 - 20.00h was significantly less than for the period 12.00 - 16.00h ($P < 0.001$ and $P < 0.01$ respectively). Points and vertical bars represent means ± s.e.m. from 5 animals in each case. Dark horizontal bar represents the dark period (food available).

Figure 21 overleaf. Data drawn from Figures 17 and 20.

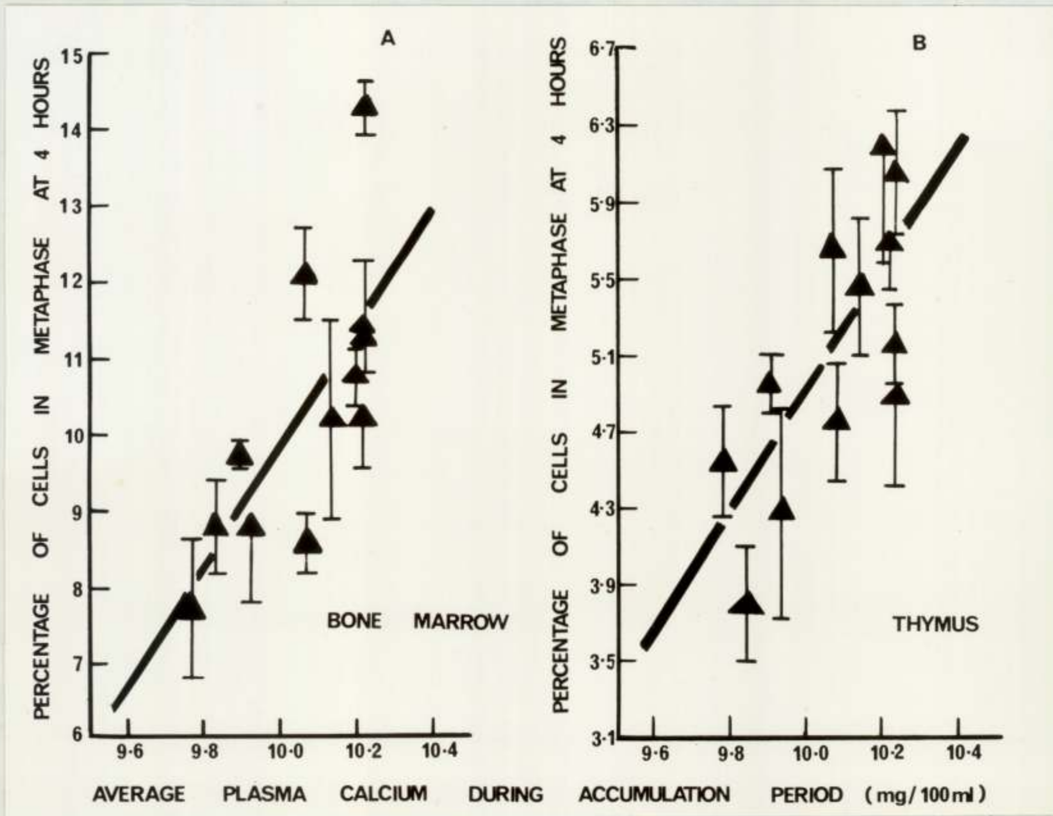


Figure 21 A/B: The relationship between the percentage of nucleated bone marrow (A) or thymus (B) cells in metaphase and the average plasma total calcium concentration over 4h accumulation periods following Colcemid administration. Points (and vertical bars) represent means ($\pm$ s.e.m. for mitosis) from 5 animals in each case. The lines of best fit are defined by (A) $y = 7.84x - 68.58$, where $r = 0.72$ and $P < 0.01$ and (B) $y = 3.21x - 27.17$, where $r = 0.74$ and $P < 0.01$

Thus the mean plasma total calcium concentration was plotted as the indepent variable against the mean mitotic index in both tissues for each of the times studied over the 24h period (Figures 19A and 19B). Lines of best fit were constructed using the method of least squares. Significant correlations were established between mitotic index in bone marrow ($r = 0.88$, $P < 0.01$) and thymus ($r = 0.82$, $P < 0.05$) and the prevailing concentration of total calcium in the plasma.

As discussed previously in full in General Methodology (Section 6.3), the use of Colcemid metaphase accumulation for the estimation of mitotic activity in tissues precludes the possibility that variations in mitotic index could reflect changes in the proportion of the cell cycle occupied by the mitotic period rather than changes in recruitment of cells into the actively cycling population. Therefore the Colcemid technique was used to estimate mitotic activity in bone marrow and thymus at 2h intervals throughout the 24h period. Marked circadian variations in cell proliferation were again observed in both tissues (Figure 20); an abrupt decline in mitotic activity commencing at 16.00 was again evident. The differences between the maximum and minimum rates of mitotic activity observed were highly significant for bone marrow ($P < 0.001$) and thymus ($P < 0.01$). Furthermore, there were again highly significant correlations ($P < 0.01$ in both cases) between mitotic activity in the two tissues and the mean plasma calcium concentrations over the accumulation periods (Figures 21A and 21B). The above correlations (Figures 19A/B and 21A/B) can be considered to constitute a further expression of the role of the calcium homeostatic system in the control of mitotic activity in rat bone marrow and thymus.

Plasma total magnesium concentrations varied by $\pm$ 0.25mg/100ml during the 24h period (Figure 23); this maximum difference, approximately 10% of the mean concentration, was statistically significant

Time of Day	Plasma Inorganic Phosphate Concentration (mg/100ml)
00.00	8.3 ± 0.3 (9)
04.00	6.9 ± 0.3 (9)
08.00	7.8 ± 0.4 (10)
12.00	7.2 ± 0.4 (10)
16.00	8.3 ± 0.4 (10)
20.00	8.5 ± 0.4 (10)

Data plotted as Figure 22 overleaf. Numbers of animals given in parentheses.

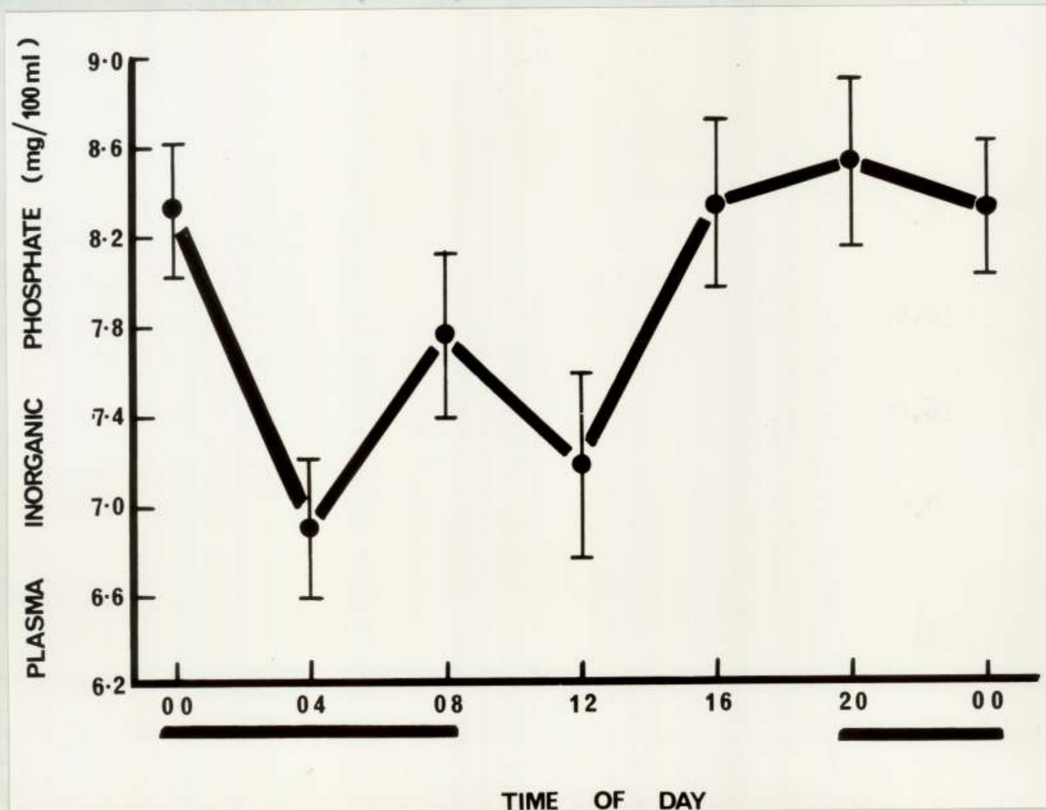


Figure 22: Variations in plasma inorganic phosphate concentration over a 24h period in the rat. The maximum plasma concentration (at 20.00) was significantly greater than the values at 04.00 and 12.00h ($P < 0.01$ in both cases). Points and vertical bars indicate mean ± s.e.m. derived from 9 - 10 animals in each case. Dark horizontal bar indicates the dark period (food available).

Time of Day	Plasma Total Magnesium Concentration (mg/100ml)
00.00	2.1 ± 0.05
04.00	2.05 ± 0.05
08.00	1.9 ± 0.05
12.00	1.85 ± 0.10
16.00	1.9 ± 0.10
20.00	2.1 ± 0.05

Data plotted as Figure 23 overleaf.

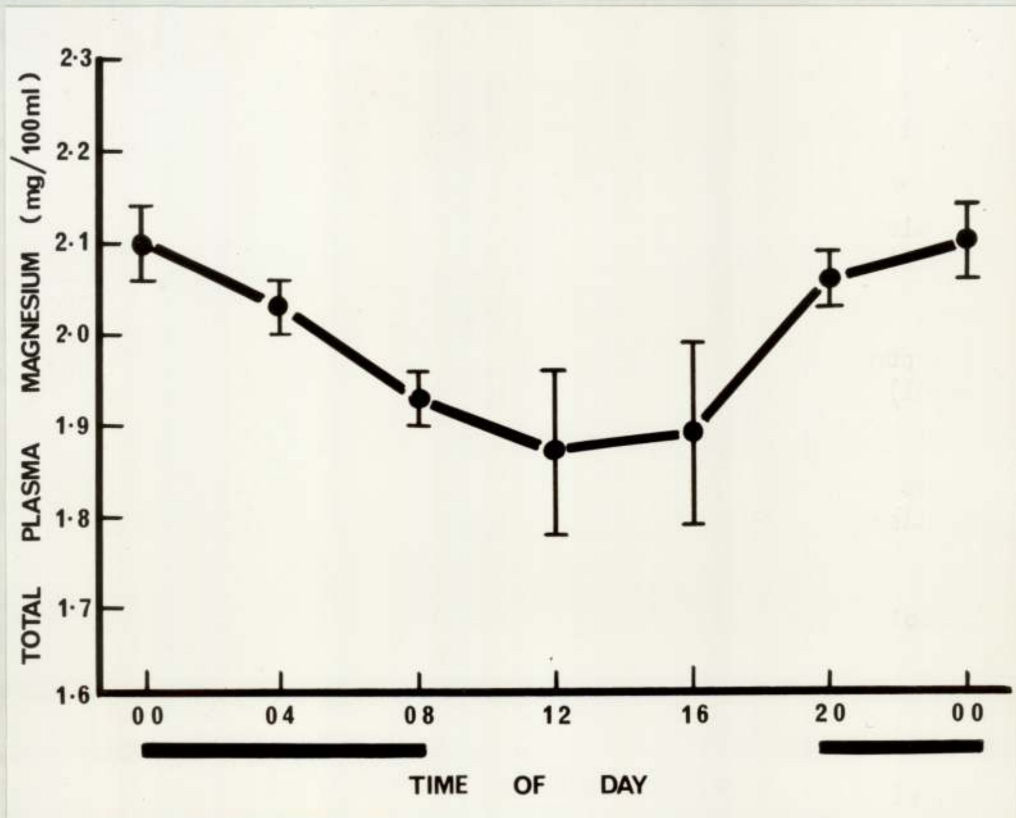


Figure 23: Variations in plasma total magnesium concentration over a 24h period in the rat. The minimum concentration of magnesium in plasma (at 12.00) was significantly lower than the peak values at 20.00 and 00.00 ($P < 0.05$ in both cases). Points and vertical bars indicate mean ± s.e.m. derived from 10 animals in each case. Dark horizontal bar indicates the dark period (food available).

	Sham-parathyroidectomized			Parathyroidectomized		
	16.00h	20.00h	P	16.00h	20.00h	P
Total calcium (mg/100ml)	10.25±0.15	9.4 ±0.11	<0.01	4.85±0.25	5.45±0.35	>0.1
Ionized calcium (mg/100ml)	5.15±0.20	4.55±0.15	<0.05	1.65±0.14	2.00±0.21	>0.1
Inorganic phosphate (mg/100ml)	8.2 ±0.24	9.0 ±0.30	>0.05	10.8 ±0.42	9.7 ±0.44	>0.1
Bone marrow mitotic index	1.26±0.04	0.89±0.08	<0.01	0.79±0.06	0.99±0.11	>0.1
Thymus mitotic index	0.86±0.10	0.59±0.05	<0.05	0.68±0.02	0.78±0.09	>0.1

Data plotted as Figure 24 overleaf. Means ±s.e.m. from 6 animals in each case.

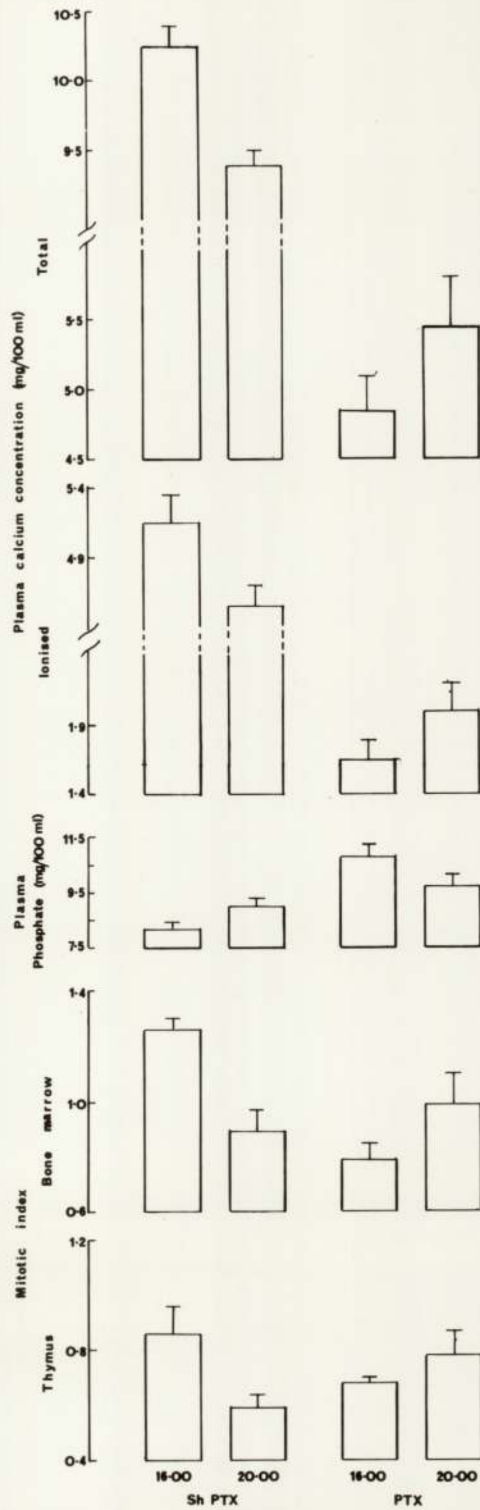


Figure 24: Abolition by PTX of the changes in plasma calcium and phosphate concentrations and in tissue mitosis occurring between 16.00 and 20.00h.

($P < 0.05$). These fluctuations were essentially opposite in phase to those of plasma calcium concentration (Figures 17 and 23), as were the changes in plasma inorganic phosphate concentration (Figures 17 and 22). The maximum and minimum concentrations of phosphate in the plasma (Figure 22) differed significantly ($P < 0.01$). The opposite nature of the variations in plasma calcium and phosphate levels suggested that the changes in their concentrations might be the result of alterations in the activity of the parathyroid gland. This hypothesis was substantiated by the observation that the abrupt decreases in plasma total and ionised calcium concentrations and tissue mitotic index which occurred at the onset of the dark period were abolished by the extirpation of the parathyroid gland (Figure 24). However, changes in parathyroid hormone activity cannot be postulated to control the observed variations in plasma magnesium concentration.

Regardless of the underlying cause of the circadian variations in plasma calcium concentration, there was no doubt that a significant decrease in plasma calcium level occurred just before the onset of darkness and the availability of food (Figure 17). Thus the physical consumption of food could not have been the trigger for this change. To determine whether anticipation of food could have in some way provoked the decrease in plasma calcium concentration, a group of animals which had been acclimatised to the controlled environment with food available _ad lib_. were compared with those which had been conditioned to the limited feeding schedule. Food intakes were measured over 2h periods in both groups to ascertain the temporal pattern of consumption (Figures 25A and 25B). The animals which had food constantly available ate substantial quantities of food before the onset of darkness (Figure 25A); indeed, approximately 30% of the total 24h consumption occurred during the light period. There was little difference in

Time Period	Food Consumption (g/100g body wt/h)	
	Free Fed	Dark Period Fed
00 - 02	0.56	0.64
02 - 04	0.61	0.92
04 - 06	0.58	0.94
06 - 08	0.21	0.49
08 - 10	0.12	-
10 - 12	0.22	-
12 - 14	0.27	-
14 - 16	0.30	-
16 - 18	0.42	-
18 - 20	0.54	-
20 - 22	1.21	1.64
22 - 00	0.72	0.83

Data plotted as Figure 25 A/B overleaf.

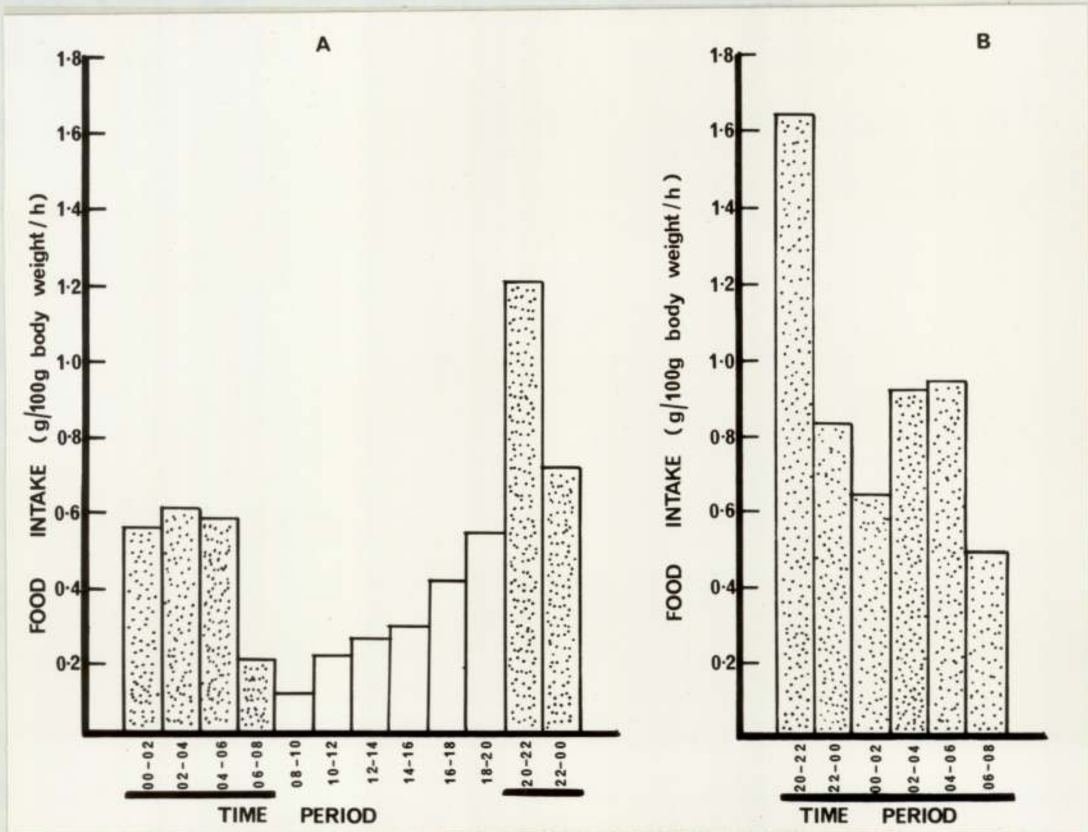


Figure 25 A/B: Food intake in rats acclimatised to a 12h light/12h darkness regimen with (A) food available <u>ad libitum</u> and (B) food available during the dark period only. The stippled colums and dark horizontal bar refer to the dark period (20.00 - 08.00).

Table 22 overleaf.

	Time of day		
	16.00 h	20.00 h	P
Plasma total calcium (mg/100 ml)	11.0 ± 0.10	10.0 ± 0.31	<0.05
Plasma ionized calcium (mg/100 ml)	5.50 ± 0.04	5.00 ± 0.10	<0.01
Plasma total magnesium (mg/100 ml)	1.95 ± 0.06	1.90 ± 0.04	>0.1
Plasma inorganic phosphate (mg/100 ml)	6.50 ± 0.32	6.20 ± 0.26	>0.5
Bone marrow mitosis (% cells in metaphase)	12.2 ± 0.74	7.6 ± 0.65	<0.01
Thymus mitosis (%cells in metaphase)	7.0 ± 0.24	4.6 ± 0.38	<0.01

Table 22: Parameters measured at 16.00 and 20.00h in rats maintained in a controlled light and temperature environment with food available ad libitum.

Animals were injected with desacetyl-N-methyl colchicine (Colcemid - dose and protocol in Methods) 4h before death. (Five animals/group, means ± s.e.m.)

the total food consumption of either group over the 24h period. Highly significant decreases in both total ($P < 0.001$) and ionised ($P < 0.001$) calcium concentrations in the plasma still occurred between 16.00 and 20.00 in the free-fed animals (Table 22). Similarly, mitotic activity in bone marrow and thymus was significantly reduced during this period ($P < 0.01$ for both tissues). There were no concomitant changes in plasma inorganic phosphate or total magnesium concentrations at this time, in agreement with the previous observations (Figures 22 and 23) in the restricted feeding situation. Anticipation of feeding did not therefore appear to provoke the changes which occurred just before the onset of darkness in plasma calcium concentration or tissue mitosis.

Discussion.

A number of situations have previously been described (General Introduction, Section 5) in which mitotic activity in rat bone marrow and thymus have been demonstrated to parallel changes in plasma calcium concentration associated with rapid growth (Perris et al. 1968), haemorrhage (Perris et al. 1971) and a variety of surgical procedures (Rixon, 1968). The present studies have shown that the observed circadian variations in tissue mitosis were closely correlated with changes in both total (Figures 19A/B and 21 A/B) and ionised calcium concentrations in the plasma. Plasma corticosteriod levels, which influence production of leucocytes and thymocytes (Dougherty, 1952; Santisteban, 1960a, b), exhibit regular rhythms with peak values occurring near the onset of darkness (Guillemin, Dear & Liebelt, 1959; Critchlow, Leibelt, Bar-Sela, Mountcastle & Lipscomb, 1963) and could therefore directly affect bone marrow and thymus mitosis. However, the precise correlations between tissue mitotic activity and plasma calcium concentration which have been described above strongly suggest that the extracellular calcium ion concentration is the major determinant of the level of mitotic activity pertinent to the present situation. Thus the calcium homeostatic system has been demonstrated to be intimately involved in the hour-to-hour control of cell proliferation in the two tissues.

The influence of the extracellular calcium environment was probably effected through the mechanism which has been extensively discussed previously (General Introduction, pp. 66 - 69 ; Perris, 1971; Whitfield et al. 1973). Thus changes in plasma calcium concentration are envisaged as having produced changes in the extracellular calcium microclimate which then resulted in variations in the numbers of cells in which DNA synthesis was initiated via the cyclic-AMP dependent

mechanism. The factors causing the primary fluctuations in plasma calcium concentration were, however, less clear.

The hormones PTH and CT are known to influence plasma levels of calcium and inorganic phosphate (Hirsch et al. 1964; Copp, 1969a) and possibly magnesium and could therefore control the observed cyclic variations in these parameters. In a variety of species including the rat (Gray & Munson, 1969; Milhaud, Perault-Staub & Staub, 1972; Swaminathan, Bates & Care, 1972), postprandial release of CT has been implicated in the physiological control of plasma calcium concentrations in young animals. However, it would be necessary to postulate a pre-prandial release of CT to explain the decreases in plasma calcium concentration observed here (Figure 17) and by Milhaud et al. (1972). Furthermore, the rats used in the present study were probably too old (8 - 9 weeks) for changes in endogenous CT secretion to produce significant changes in plasma calcium levels (Copp, 1969b). The mechanism producing the changes was not activated by anticipation of feeding (Table 22). However, it is possible that the precise changes in illumination could have acted as Zeitgebers controlling a rhythm of CT and/or PTH activity. Such a rhythm would have to anticipate the onset of darkness to produce the observed changes in plasma calcium concentration.

The opposite phases of the variations in plasma calcium and inorganic phosphate concentrations suggested that changes in PTH activity might be the most important control factor. This hypothesis was strengthened by the observation that extirpation of the parathyroid glands abolished the rapid shifts in plasma calcium concentration and tissue mitosis which had occurred between 16.00 and 20.00 in the intact animal. Undoubtedly, circadian changes in CT activity exist and may be necessary for control of post-prandial hypercalcaemia in younger rats during normal feeding in the hours of darkness as described by Milhaud et al.

(1972). However, these authors also demonstrated that a pre-prandial depression in plasma total calcium concentration still occurred in thyroidectomised animals and could therefore be due to a decrease in the activity of the parathyroid gland.

A number of other hormones, including erythropoietin (Results Chapter 2; Hunt & Perris, 1973) and, possibly, adrenaline (Kenny, 1964; Fischer, 1973) may cause perturbations in plasma calcium concentration. It is unlikely that erythropoietin could effect the observed changes in plasma calcium concentration, for it is probable that plasma erythropoietin titres would be highest during the dark period which is the time of maximum activity of the rat (Besch, 1969). It has been established in man that erythropoiesis and urinary erythropoietin excretion are maximal when oxygen demand is highest (Adamson, Alexanian, Martinez & Finch, 1966; Lockner, 1966). Since plasma calcium concentrations are increased by increasing erythropoietin activity (Results, Chapter 2; Hunt & Perris, 1973), any interaction of the hormone with the calcium homeostatic system at the onset of darkness must have been overriden by a more dramatic stimulus. Any significance for glucagon and adrenaline in the aetiology of the observed plasma calcium shifts is not clear without further evidence of the effectiveness of the changes in their circulating levels in affecting calcium homeostasis.

Circadian changes in plasma calcium concentrations in man have been postulated (Jubiz et al.1972) to be secondary to the variations in plasma protein levels associated with postural changes (Husdan, Rapoport & Locke, 1973). In the rat these changes would be small because of its supine posture and probably do not significantly affect plasma calcium levels, since the large decrease in ambient calcium in the plasma seems to depend ultimately on the presence of the parathyroid gland (Figure 24). Postural changes in humans do not affect plasma

ionised calcium levels (Husdan et al. 1973); thus postural shifts are unlikely to produce the observed changes in this ion in the rat. The abolition of this decrease by PTX could be considered to indicate that the plasma levels of calcium in the aparathyroid rat are so low as to be incapable of being further depressed. However there is evidence that aparathyroid rats may be rendered further hypocalcaemic, e.g. following haemorrhage (Results, Chapter 3, pp. 257- 258 ; Perris et al. 1971). It is possible, but unlikely, that the time scale of circadian plasma calcium changes was merely offset and therefore not observed between 16.00 and 20.00, though this had obviously not occurred in sham-operated controls (Figure 24).

It is clear from the results presented above that the presence of the parathyroid gland is essential for the manifestation of the pre-prandial decrease in plasma calcium concentration. In addition, the relationships between plasma calcium concentration and mitotic activity in bone marrow and thymus which have been demonstrated can be added to the body of evidence which emphasises the importance of the calcium homeostatic system in the physiological control of cell division in these tissues.

Summary.

Circadian changes in plasma total and ionised calcium, inorganic phosphate and total magnesium concentrations and bone marrow and thymus mitotic activity have been observed in rats conditioned to a constant environment. The changes in tissue mitosis have been shown to closely parallel the shifts in plasma calcium concentration.

Pre-prandial decreases in plasma calcium concentrations and tissue mitotic activity were abolished by parathyroidectomy, suggesting that the circadian variations in plasma calcium levels might be dependent upon changes in parathyroid hormone activity.

The evidence infers that hour-to-hour variations in the systems controlling calcium homeostasis determine the levels of mitosis in rat bone marrow and thymus unless overriden by more specific mitotic stimuli.

These observations imply that all experiments in which any of the parameters which have been shown to undergo circadian variations are to be measured should be terminated at the same time of day and carefully controlled. This approach was adopted in subsequent studies.

CHAPTER 2.

Interrelationships between exogenous erythropoietin and calcium homeostasis.

Portions of the studies reported in this Chapter were performed by Dr. A.D. Perris at the Division of Biology, National Research Council, Ottawa, Canada, and I would like to express my gratitude for his permission to include these data here.

The experiments summarised in Figures 26, 28, 30 and 31 were performed by A.D.P. alone; Figures 32, 33, 35 and Table 23 resulted from joint efforts and Figures 27, 29 and 34 are derived from the present author's investigations. Thus approximately 50% of the observations reported here were made by each party.

Some of these studies have been published (N.H. Hunt & A.D. Perris, (1973). Erythropoietin-induced changes in plasma calcium and bone marrow mitosis in the rat. J. Endocr. 56, 47-57).

Introduction.

A number of factors which influence erythropoietic activity in rats have been discussed previously (General Introduction, Sections 2 and 5). The most important of these in the in vivo situation are the hormone EPO (Gordon & Zanjani, 1970) and the concentration of ionised calcium in the extracellular fluids (Perris, 1971; Whitfield et al., 1973c). These factors appear to constitute control systems rather than non-specific influences since they have been demonstrated to act directly upon erythroid elements (Goldwasser, 1966; Morton, 1968; Morton et al., 1968; see also Chapter 5), in contrast to many other agents which act indirectly via one or other of these major control systems (Gordon & Zanjani, 1970; Fisher, 1972; see also General Introduction, Section 2.12 and Results, Chapter 4).

While the competence to synthesize and secrete EPO has long been established as an essential prerequisite for the maintenance of normal red cell production (Jacobson et al., 1957b; Stohlman, 1962; Gordon et al., 1967) a similar absolute requirement for a functional parathyroid gland has been demonstrated only recently (Perris, 1971; Perris & Whitfield, 1971; Rixon & Whitfield, 1972a). The importance of this gland is shown by the depressions of the activity (Perris & Whitfield, 1971) and cellularity (Rixon & Whitfield, 1972a) of erythroid tissue and of the ability to rapidly replace lost red cells (Perris et al., 1971) which are evident after parathyroidectomy. These lowered erythropoietic indices have all been shown to reflect the reductions in mobilised body calcium consequent upon parathyroid removal. Furthermore, in several other situations in which plasma ionised calcium increases, e.g. during rapid growth (Perris et al., 1968), after PTH or $CaCl_2$ injections (Perris & Whitfield, 1971) or as a result of infradian variation (Chapter 1), parallel surges in erythropoiesis and/or bone marrow mitotic activity have been demon-

strated.

In view of these relationships it seemed pertinent to investigate the possibility of interactions between EPO and the calcium homeostatic system. The studies reported here were therefore designed to determine:

(i) If exogenous EPO produced shifts in plasma calcium concentration, and if so,

(ii) whether such fluctuations were parallelled by changes in bone marrow mitotic activity, and finally

(iii) to determine whether these activities of exogenous EPO were dependent upon the presence of the parathyroid gland. The effects of endogenous EPO were later investigated in similar ways (Chapters 3 and 4).

Methods.

Bone marrow mitotic activity and plasma concentrations of total and ionised calcium were measured in rats at various intervals after the administration of EPO. Different amounts of Step I (0.4 or 0.6 units/mg) or Step III(3.5 units /mg) ovine EPO (Connaught Laboratories) were dissolved in 0.9% saline and administered as single 0.5ml injections subcutaneously in the dorso-thoracic region of male albinos. Animals of both the Sprague-Dawley and Wistar strains, weighing 180 - 220g, were used in these studies. No significant differences in the responses of the two strains to EPO were noted.

Mitotic activity in bone marrow cells was assessed by determination of the mitotic index and by use of the metaphase arresting agent Colcemid. Suspensions and smears of bone marrow were prepared, fixed, stained and scored as before. Detailed discussions of these procedures have been given (General Methodology, Section 6). The concentrations of total and ionised calcium in plasma were measured as described previously (General Methodology, Section 5).

Dose of EPO (u/100g body weight)	Plasma Calcium Concentration (mg/100ml)	
	Step I	Step III
0	10.4 $\pm$ 0.2 (28)	10.25 $\pm$ 0.1 (10)
2.5	11.1 $\pm$ 0.1 (5)	—
5	11.3 $\pm$ 0.2 (10)	10.75 $\pm$ 0.3 (5)
10	11.5 $\pm$ 0.1 (9)	11.1 $\pm$ 0.1 (9)
25	11.6 $\pm$ 0.1 (9)	11.7 $\pm$ 0.1 (5)

Data plotted as Figure 26 overleaf. Numbers of animals indicated in parentheses.

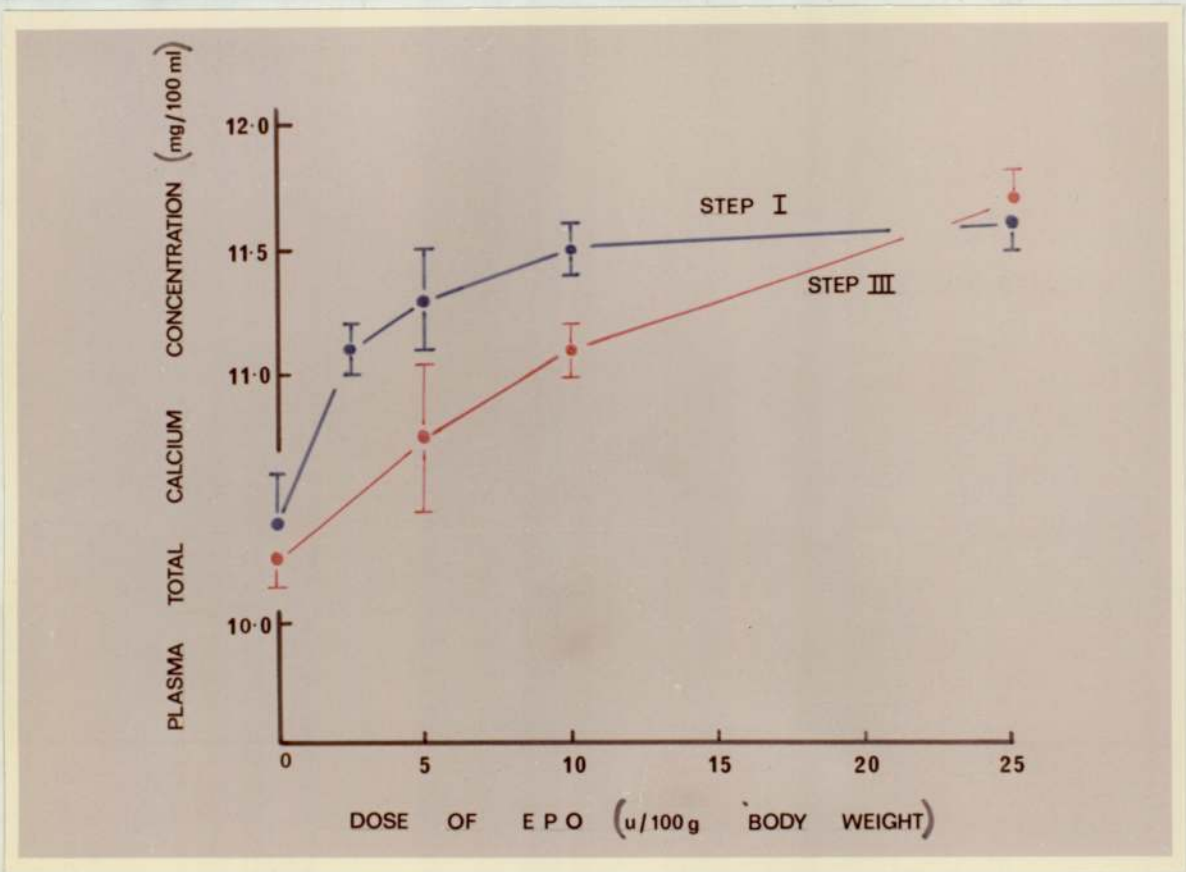


Figure 26: Effect of EPO on plasma calcium concentrations in rats. Plasma levels were measured 24h after injection of various doses of Step I or Step III EPO (protocol in Methods). Controls received saline. All doses of Step I EPO caused a significant increase in calcium concentration ($P < 0.001$). The Step III preparation gave significant calcium elevations at the 5 ($P < 0.05$), 10 and 25u/100g ($P < 0.001$) doses. The increases in plasma calcium concentrations above control values induced by equal doses of the two EPO preparations were not significantly different ($P > 0.2$ in all cases) Values are means ± s.e.m. from 5 - 28 rats.

The action of exogenous EPO was also investigated in aparathyroid (both PTX and TPTX) animals. The hormone was administered on the 5th post-operative day to animals which had been successfully deprived of their parathyroid glands (see General Methodology, Section 4.2 for operation description and criteria) and maintained for 2 days on a calcium-deficient diet (Kenny & Munson, 1959).

To test whether the ability of the ovine Step I EPO extract to increase plasma calcium concentration was due to innate activity of the hormone or to impurities present in the preparation the EPO molecule was specifically inactivated by mild acid hydrolysis according to the method of Rambach, Shaw, Cooper & Alt (1958). The Step I extract was dissolved in either 5ml of distilled water or the same volume of 0.005M H_2SO_4 and then incubated at 80°C for 1h. The treated EPO was subsequently dialysed against 2 litres of distilled water (changed twice daily) for 4 days. The resultant protein residues were redissolved in distilled water and lyophilised. The lyophilised powders were then dissolved in saline and injected as described above. As a further control some Step I samples were dissolved in cold water and immediately lyophilised without subsequent heating or dialysis. In another experiment, animals were injected with extremely pure preparations of ovine plasma albumin (Sigma Chemicals) or α-globulin (Schwartz Mann Co.) to determine whether administration of plasma proteins _per se_ could mimic the effects of EPO.

Results.

The administration of increasing doses of Step I EPO produced progressive elevations in plasma calcium concentrations 24h later (Figure 26). The increment in plasma total calcium concentration was maximal and of the order of 1mg/100ml in the dose range 10 - 20u/100g body weight. Approximately half of these calcium increases were in the ionised calcium fraction (values not shown in Figure 26, but see

Time After Injection (hours)	Plasma Calcium Concentrations (mg/100ml)	
	Total	Ionised
0	10.1 ± 0.25 (6)	5.2 ± 0.2 (6)
24	11.2 ± 0.2 (6)	5.7 ± 0.1 (6)
48	10.6 ± 0.1 (6)	5.3 ± 0.1 (6)
72	10.5 ± 0.2 (6)	5.3 ± 0.2 (6)

Data plotted as Figure 27 overleaf.

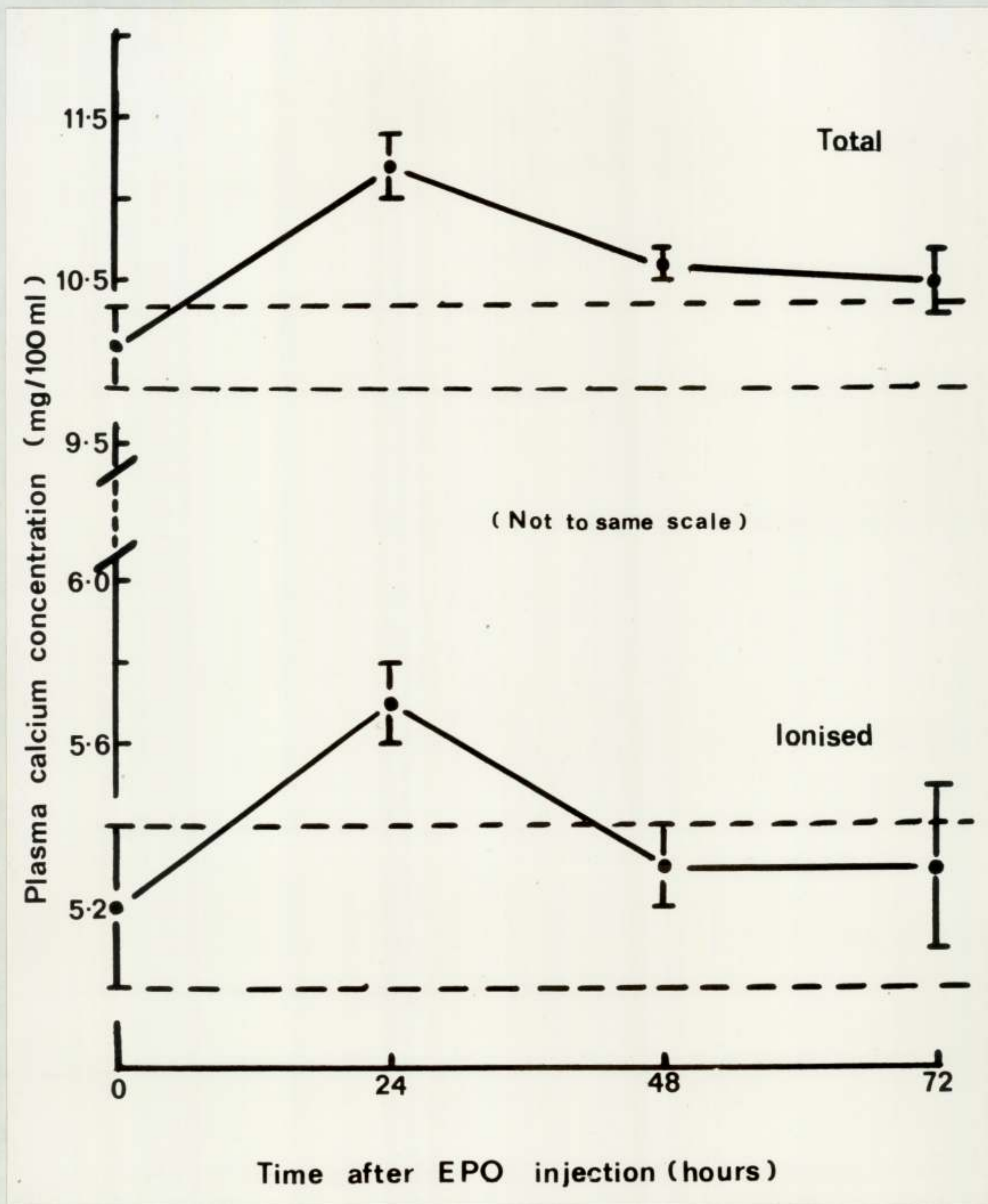


Figure 27: Effect of EPO on plasma calcium concentrations in rats. Plasma calcium levels were measured at various times after injection of 10u/100g Step I EPO (see Methods for protocol). The increments in both total and ionised calcium at 24h were significant ($P < 0.001$ and $P < 0.05$ respectively). Values are means ± s.e.m. from 6 animals in each case.

Treatment	Plasma Calcium Concentrations (mg/100ml)	
	Total	Ionised
Saline	10.4 ± 0.0	5.15 ± 0.05
EPO (cold water)	11.4 ± 0.3	5.85 ± 0.05
EPO (hot water)	11.0 ± 0.1	5.55 ± 0.10
EPO (hot acid)	10.1 ± 0.2	4.85 ± 0.15

Data plotted as Figure 28 overleaf. All values are derived from 4 rats.

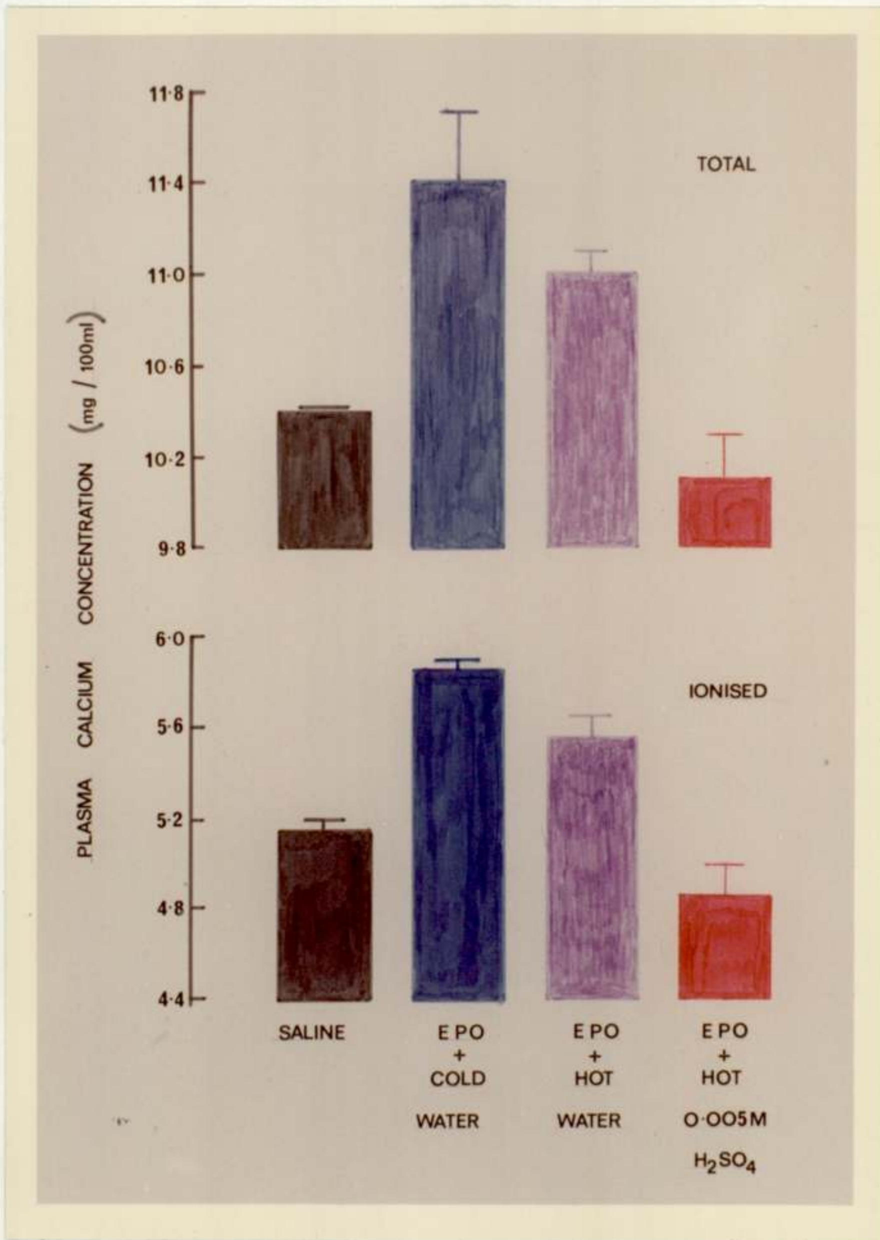


Figure 28: Effect of mild acid hydrolysis on the ability of EPO to cause hypercalcaemia in rats. Quantities of Step I EPO were treated as described in Methods. Fractions of such preparations which had contained 20u of EPO were dissolved in saline and injected (protocol in Methods) into 200g rats. Plasma calcium concentrations were measured 24h later. EPO + cold water produced significant increases in plasma total ($P<0.01$) and ionised ($P<0.001$) calcium concentrations. Hot water incubation did not significantly decrease these abilities of EPO ($P>0.1$ in both cases). Mild acid hydrolysis destroyed the calcium-elevating properties of EPO (compare columns 1 and 4, 5 and 8; $P>0.1$) Columns and vertical bars are means ± s.e.m. from 4 rats in each case.

Treatment	Plasma Calcium Concentration (mg/100ml)		Bone Marrow Mitotic Index
	Total	Ionised	
Saline	10.5±0.1 (10)	5.40±0.05 (5)	1.25±0.05 (10)
Step I EPO (20u = 33mg)	11.3±0.1 (5)	5.90±0.10 (5)	2.20±0.2 (5)
Albumin (33mg)	10.25±0.1 (10)	5.30±0.05 (5)	1.25±0.1 (9)
α-Globulin (33mg)	10.1 ±0.2 (10)	5.20±0.10 (5)	1.20±0.1 (10)

Data plotted as Figure 29 overleaf. Numbers of animals indicated in parentheses.

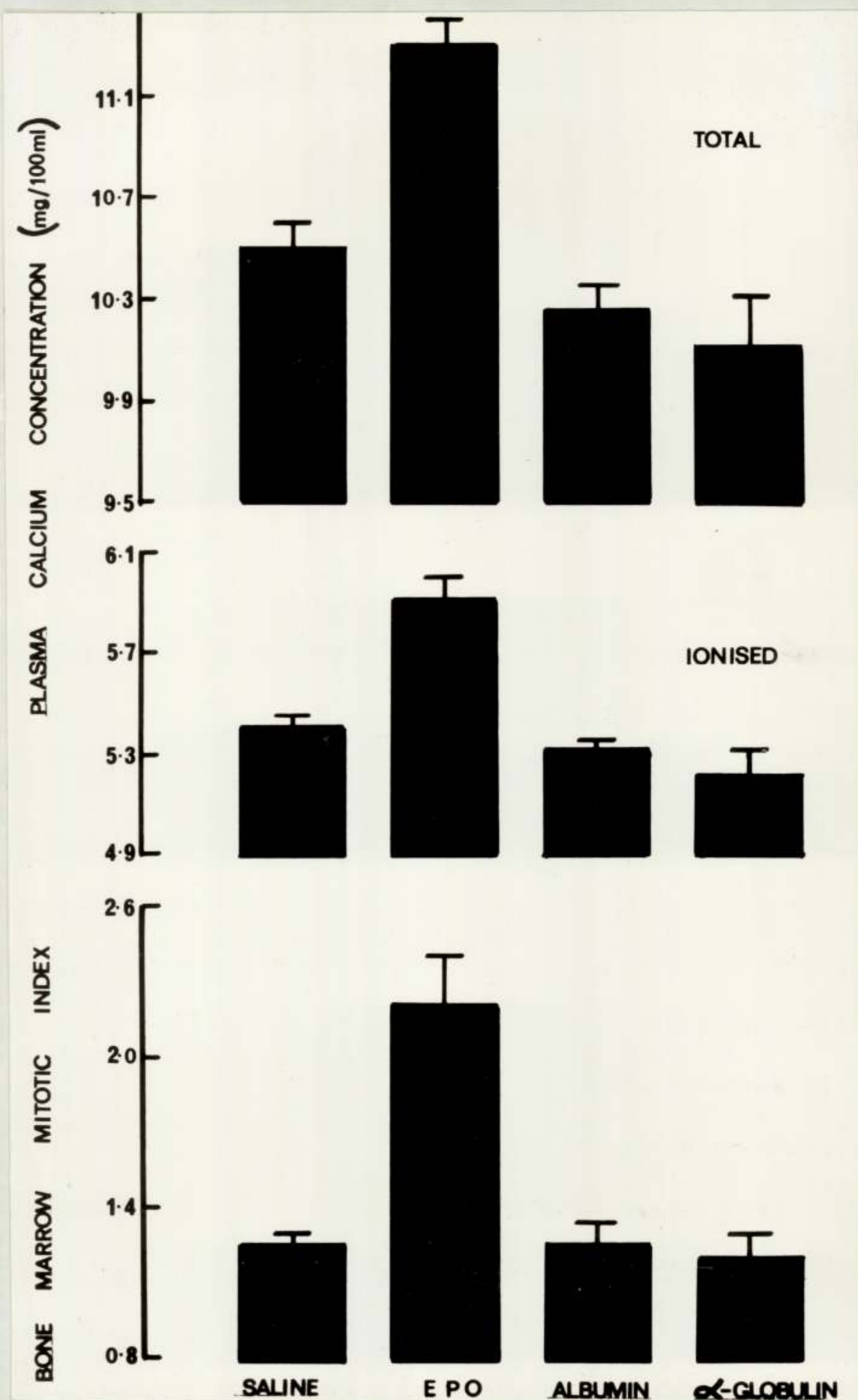


Figure 29: Effects of EPO, albumin and α-globulin on plasma calcium concentration and bone marrow mitotic index of rats. The proteins (16.5mg/100g body wt.) were injected s.c. in the dorso-thoracic region. Plasma calcium levels and bone marrow mitotic index were assessed 24h later. Step I EPO caused significant increases in plasma total ($P<0.01$) and ionised ($P<0.05$) calcium concentrations and in mitotic index ($P<0.05$). Albumin and globulin did not significantly affect these parameters ($P>0.1$ in all cases). Columns and vertical bars represent mean + s.e.m. derived from 5 - 10 animals in each case.

Figures 27 and 32). This pronounced calcium elevation had decreased by 48h after injection of Step I EPO and was not evident at 72h (Figure 27).

When the more highly purified Step III preparation was given to normal animals plasma calcium concentration again rose at 24h. The degrees of hypercalcaemia produced by the two preparations were similar at all dose levels tested, suggesting that this calcium-elevating ability was not due to impurities in the Step I ovine extract. This conclusion was reinforced by the experiments summarised in Figures 28 and 29. Dissolution of the Step I EPO preparation in cold water, followed by immediate lyophilisation, had no effect upon its ability to raise plasma total and ionised calcium concentrations (compare Figures 26 and 28). Incubation at 80°C for 1h following dissolution in water, with subsequent dialysis and lyophilisation, likewise caused only a slight reduction in the hypercalcaemic properties of the Step I extract. In marked contrast, mild acid hydrolysis at 80°C, followed by the preparative procedures, completely abolished any increment in plasma total and ionised calcium concentration 24h after injection of the treated EPO (Figure 28). This hydrolysis precedure has been demonstrated to desialate the EPO molecule and thereby destroy its ability _in vivo_ to increase radioiron incorporation into red cell precursors (Rambach _et al._, 1958). Thus the present observations strongly imply that the ability to elevate plasma calcium concentrations is a specific property of the EPO molecule. As a further investigation of this possibility, parallel groups of animals were injected with equal amounts of the plasma proteins albumin and α-globulin and with Step I EPO. The EPO preparation significantly increased plasma calcium concentrations (both total and ionised) and bone marrow mitotic index at 24h, whereas the plasma proteins did not significantly alter these parameters (Figure 29).

Dose of EPO (u/100g body wt.)	Bone Marrow Mitotic Activity 24h After EPO (% Nucleated Cells in Metaphase at 6h After Colcemid Injection)
0	14.5 ± 0.6 (20)
2.5	16.7 ± 0.7 (9)
5	17.4 ± 0.7 (9)
10	19.7 ± 0.9 (20)

Data plotted as Figure 30 overleaf. Numbers of animals indicated in parentheses.

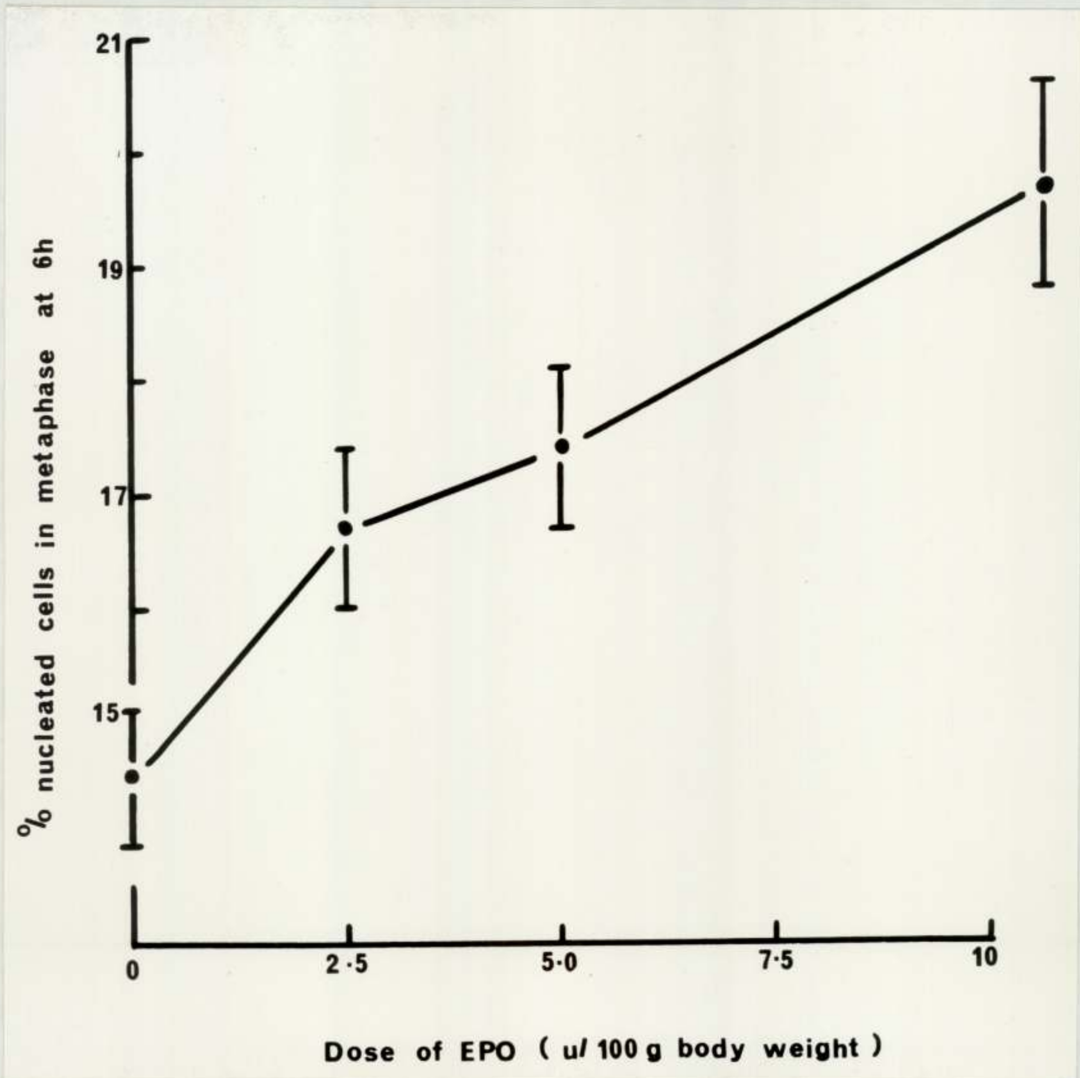


Figure 30: Effect of EPO upon bone marrow mitotic activity in rats. Colcemid (protocol and dose in Methods) was administered 18h and 21h after various doses of Step I EPO. Mitotic activity was assessed at 24h. At this time mitosis was significantly increased at all dose levels. The level of significance increased from $P < 0.05$ to $P < 0.001$ with increasing dose. Points and vertical bars represent means ± s.e.m. from 9 - 20 rats in each case.

Dose of Step I EPO (u/100g body wt.)	Bone Marrow Mitotic Index at 24h.
0	1.15 ± 0.1 (14)
2.5	1.60 ± 0.1 (7)
5	1.85 ± 0.2 (8)
10	1.95 ± 0.1 (14)

Data plotted as Figure 31 overleaf. Numbers of animals given in parentheses.

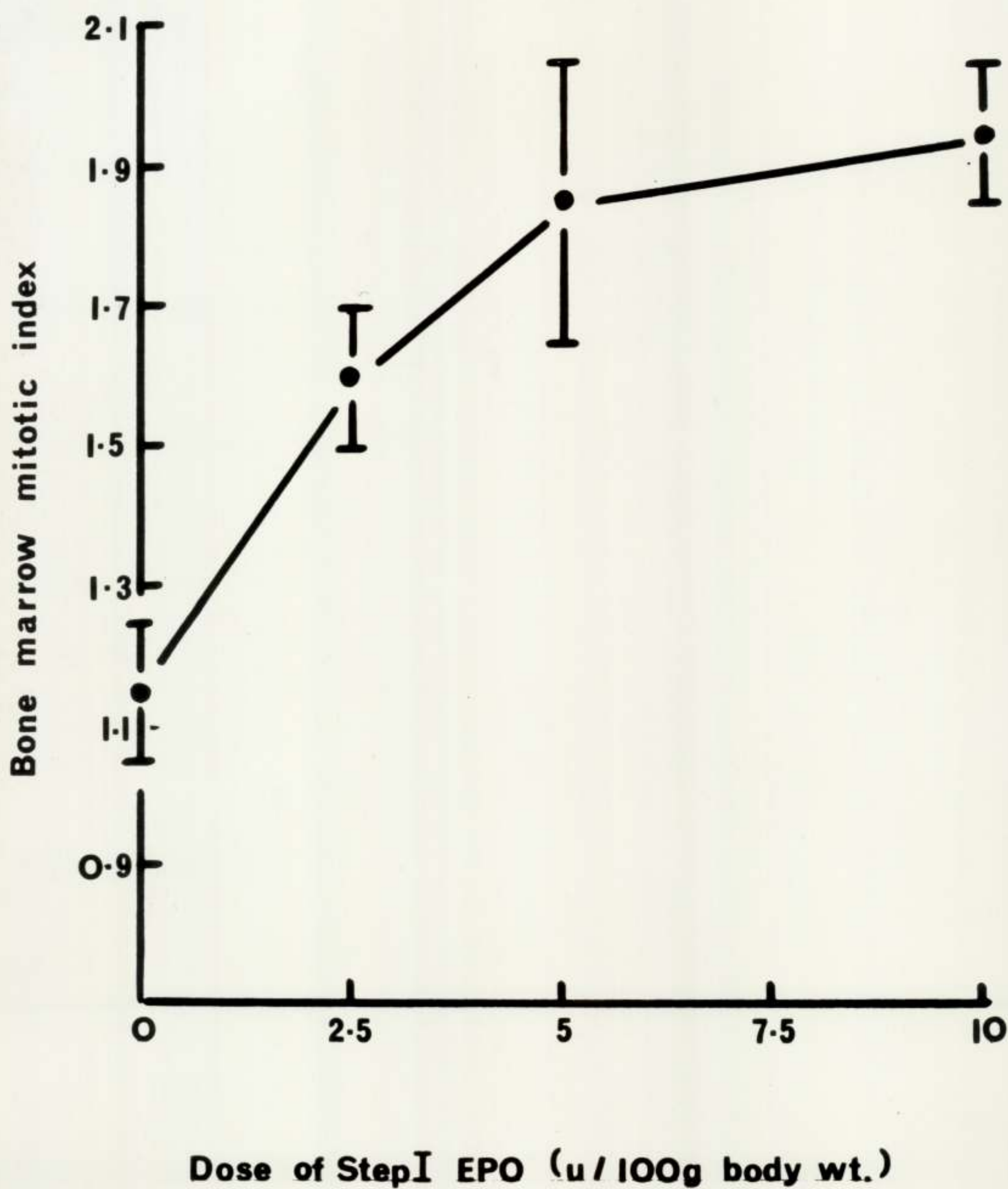


Figure 31: Effect of increasing doses of EPO upon bone marrow mitotic index in rats. Mitotic index was assessed 24h after injection (protocol in Methods) of Step I EPO. Significant elevations were observed at all dose levels ($P < 0.001$). Points and vertical bars represent mean ± s.e.m. from 7 - 14 animals in each case.

Treatment	Plasma Calcium Concentration (mg/100ml)	
	Total	Ionised
Normal + saline	10.4 ± 0.1 (28)	5.20 ± 0.05 (10)
Normal + EPO	11.5 ± 0.1 (22)	5.80 ± 0.05 (10)
TPTX + saline	6.2 ± 0.2 (20)	2.20 ± 0.15 (5)
TPTX + EPO	6.6 ± 0.2 (18)	2.30 ± 0.30 (4)
PTX + saline	6.4 ± 0.2 (9)	2.90 ± 0.20 (3)
PTX + EPO	6.0 ± 0.3 (9)	2.50 ± 0.25 (3)

Data plotted as Figure 32 overleaf. Numbers of animals indicated in parentheses.

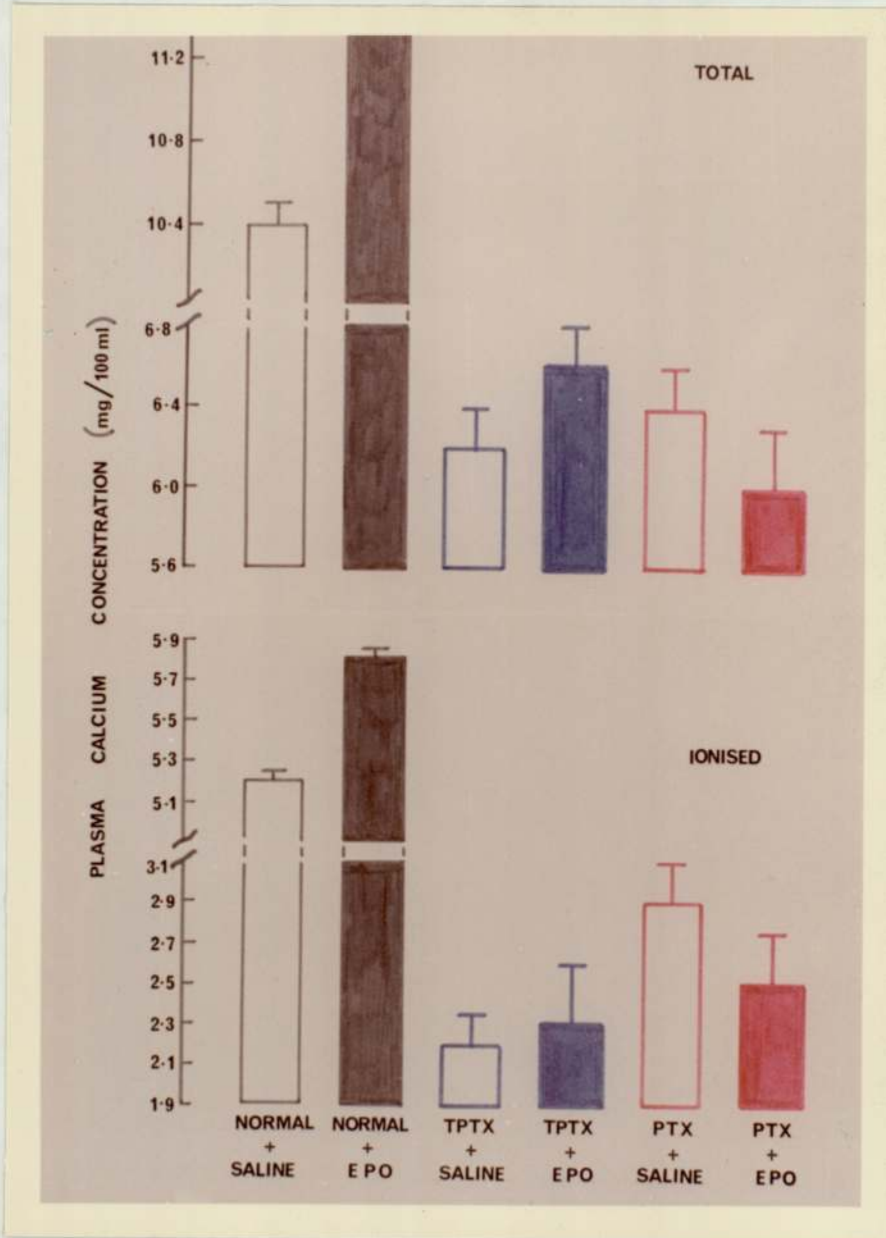


Figure 32: Effect of EPO on plasma calcium concentrations in normal and aparathyroid rats. Step I EPO (10u/100g) was injected at 0h (protocol in Methods) and plasma total and ionised calcium concentrations measured at 24h. EPO had no significant effects ($P > 0.1$) on plasma calcium levels in TPTX or PTX rats but produced a significant increase in both total ($P < 0.001$) and ionised ($P < 0.001$) calcium in intact rats. Columns and bars represent mean $\pm$ s.e.m. from 9-28 (total calcium) and 3-10 (ionised calcium) animals in each case.

Treatment	Bone Marrow Mitotic Activity 24h After EPO (% Nucleated Cells in Metaphase 6h After Colcemid)
Normal + saline	15.5 ± 0.8 (4)
Normal + EPO	20.3 ± 0.9 (5)
TPTX + saline	9.7 ± 0.6 (5)
TPTX + EPO	11.5 ± 0.6 (5)

Data plotted as Figure 33 overleaf. Numbers of animals indicated in parentheses.

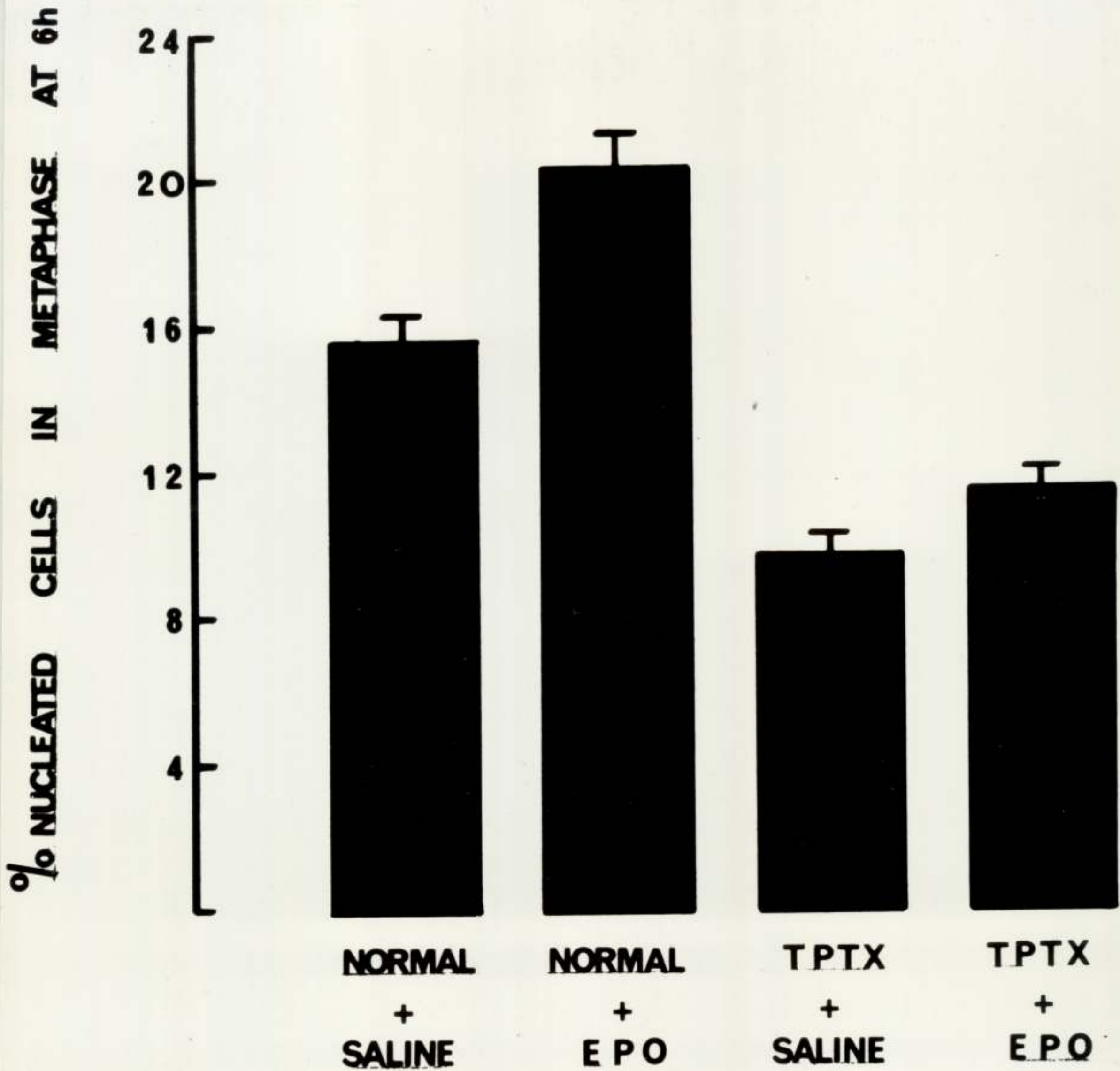


Figure 33: Effect of EPO on bone marrow mitotic activity in normal and TPTX rats. Colcemid (dose and protocol in Methods) was administered 18 and 21h after Step I EPO (10u/100g body wt.). Mitotic activity was assessed at 24h. EPO significantly increased mitosis in normal rats ($P < 0.01$) but not in TPTX animals ($P > 0.05$). Columns and vertical bars are means $\pm$ s.e.m. from 4 - 5 animals in each case.

Treatment	Bone Marrow Mitotic Index 24h After Injection
Normal + saline	1.2 $\pm$ 0.1 (14)
Normal + EPO	1.8 $\pm$ 0.1 (8)
TPTX + saline	0.95 $\pm$ 0.1 (5)
TPTX + EPO	1.05 $\pm$ 0.2 (5)
PTX + saline	0.9 $\pm$ 0.1 (3)
PTX + EPO	0.9 $\pm$ 0.1 (3)

Data plotted as Figure 34 overleaf. Numbers of animals indicated in parentheses.

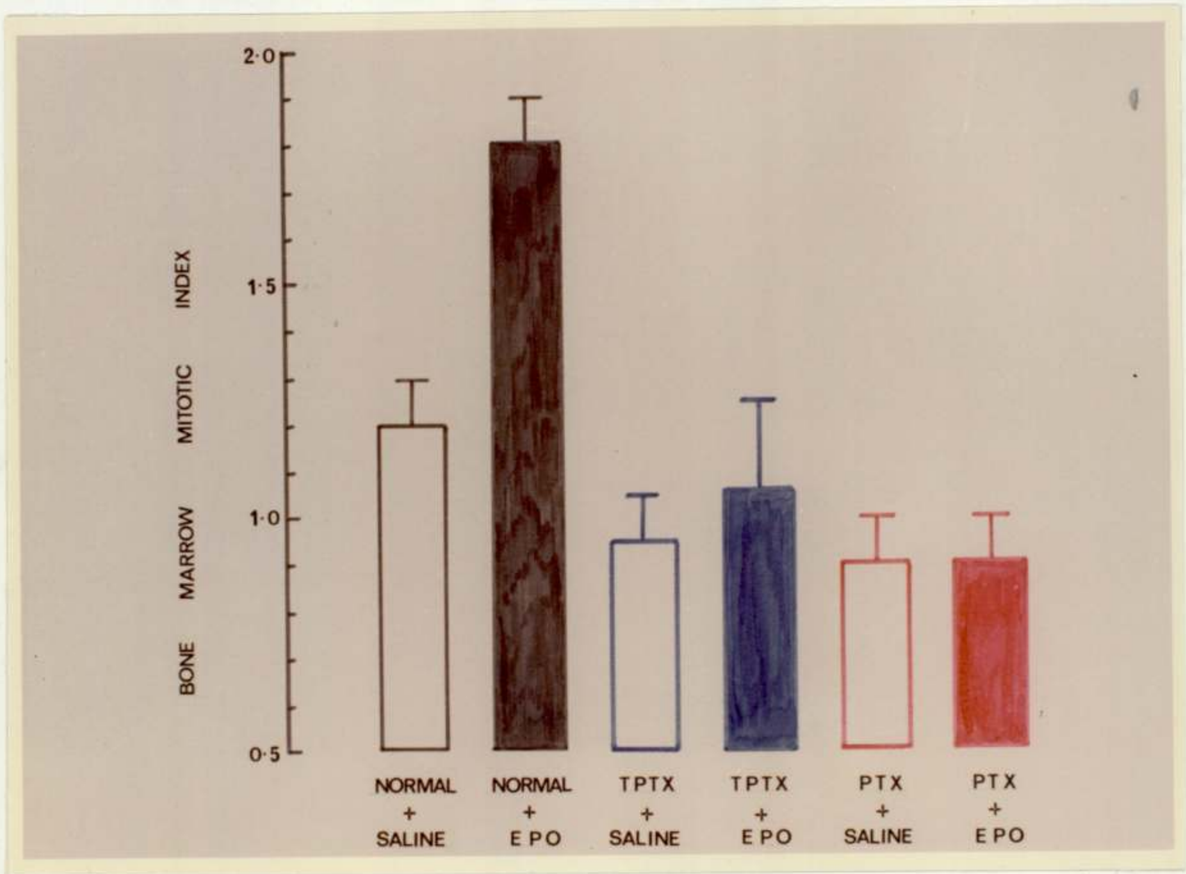


Figure 34: The effect of EPO on bone marrow mitotic index in normal and aparathyroid rats. Mitotic index was determined 24h after injection of 10u/100g Step I EPO or saline (controls). EPO produced a significant ($P < 0.01$) increase over controls in bone marrow mitotic index in intact rats but not in TPTX or PTX (aparathyroid) animals. Columns and vertical bars represent mean ± s.e.m. from 3 - 14 animals in each case.

Time After EPO Injection (h)	Plasma Total Calcium Concentration (mg/100ml)
0	10.1 ± 0.1 (24)
2	10.3 ± 0.1 (10)
3	10.2 ± 0.3 (5)
6	10.0 ± 0.1 (14)
16	11.1 ± 0.1 (6)
20	11.2 ± 0.1 (6)
24	11.5 ± 0.1 (9)

Data plotted as Figure 35 overleaf. Numbers of animals indicated in parentheses.

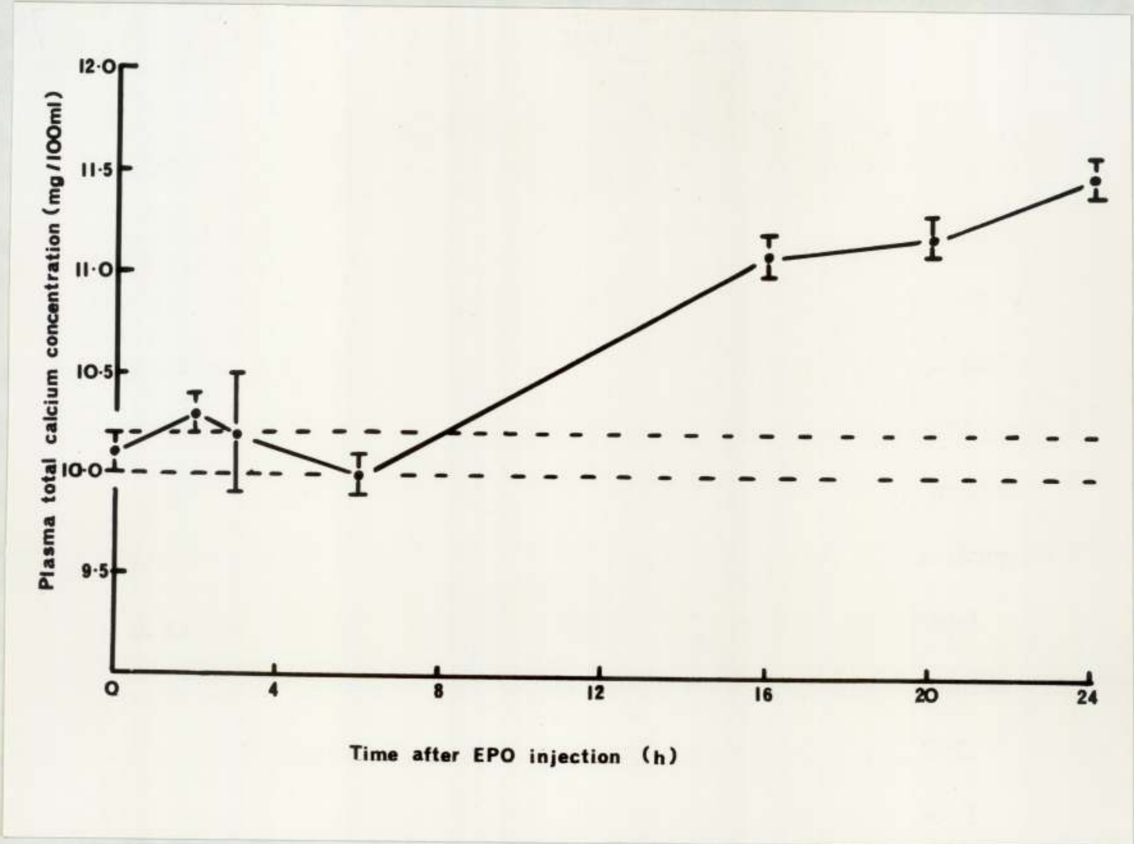


Figure 35: Effect of EPO on plasma calcium concentrations in the rat. Step I EPO (10u/100g body wt.) injected at 0h produced no significant changes in plasma calcium levels compared with controls at 2, 3 or 6h ($P > 0.1$). Values at later times were significantly higher than normal ($P < 0.01$). The horizontal line and shaded area represent the mean plasma total calcium concentration of 24 control rats ± s.e.m. Points and vertical bars represent means ± s.e.m. derived from 5-14 rats in each case.

Mitotic activity in the bone marrow was increased 24h after EPO (Figures 30 and 31) in parallel with plasma calcium concentration. The significant ($P<0.001$) increments in mitosis produced by 10u/100g EPO were of the same order of magnitude as that produced by PTH-induced hypercalcaemia (Perris et al., 1967, 1971; Rixon, 1968). In contrast to its activity in the whole animal, EPO was incapable of stimulating bone marrow cellular proliferation in aparathyroid rats at 24h (Figures 32 and 33). Furthermore, the hormone was incapable of producing increases in plasma calcium concentrations in TPTX or PTX animals 1 day after administration (Figure 34). Thus the presence of the parathyroid gland appeared to be a prerequisite for the expression of the hypercalcaemic and mitogenic properties of exogenous EPO.

The parathyroid-mediated hypercalcaemia which occurred 24h after EPO injection (Figures 26, 27 and 32) could have resulted from supranormal synthesis and release of PTH after an initial hypocalcaemic episode. However, no significant decrease in plasma total or ionised calcium concentrations was observed after EPO injection into either normal or TPTX rats (Figure 35, Table 23).

The effects of exogenous EPO upon plasma calcium concentrations and bone marrow mitosis in intact, TPTX and PTX rats are summarised in Table 24.

Table 23 overleaf.

Time After EPO Injection (h)	Plasma Calcium in Normal Rats (mg/100ml)		Plasma Calcium in TPTX Rats (mg/100ml)	
	Total	Ionised	Total	Ionised
0	10.1 ± 0.1 (24)	5.30 ± 0.10 (16)	5.0 ± 0.2 (4)	2.15 ± 0.10 (6)
3	10.2 ± 0.3 (5)	5.40 ± 0.10 (5)	5.0 ± 0.2 (8)	2.10 ± 0.10 (6)
6	10.0 ± 0.1 (14)	5.00 ± 0.20 (10)	5.0 ± 0.3 (4)	2.10 ± 0.10 (6)

Table 23: The effect of EPO on plasma calcium levels shortly after injection in normal and TPTX rats. Step I EPO (10u/100g body wt.) was injected (protocol in Methods) into normal and TPTX animals. There was no significant change in plasma total or ionised calcium concentrations within 6h ($P > 0.1$). Values are means ± s.e.m. Numbers of animals indicated in parentheses.

Discussion.

The demonstrations that exogenous EPO can increase plasma calcium concentrations and thereby enhance bone marrow cell proliferation in intact rats (Figures 26 - 31, Table 24), but not in aparathyroid animals (Figures 32 - 34, Table 24), confirms the significance of the parathyroid gland in the control of bone marrow mitotic activity and erythropoiesis, which had been suggested by earlier studies (Perris, 1971; Perris & Whitfield, 1971; Perris _et al._, 1971). Since the doses of EPO which were investigated in the present study were within physiological ranges (c.f. Fried, Johnson & Heller, 1970) it is tempting to postulate that these observations have significance for the response of the intact animal to erythropoietic challenge, e.g. haemorrhage (Perris _et al._, 1971). Furthermore, the necessity of the presence of a functional parathyroid gland for the maintenance of normal erythropoiesis (Perris & Whitfield, 1971; Rixon & Whitfield, 1972a) leaves no doubt that calcium homeostasis is closely related to the regulation of erythropoietic activity in normal situations.

EPO has been shown to elevate both bone marrow mitotic index (Figures 31 and 34, Table 24) and the rate of entry of marrow cells into mitosis as assessed by metaphase accumulation with Colcemid (Figures 30 and 33, Table 24). Thus it seems certain that the action of the hormone is to increase the mitotic activity of bone marrow cells, i.e. to raise the number of cells undergoing mitosis at the time of assessment (24h after EPO administration). Although no measurement was made of radioactive iron incorporation into erythrocyte precursors or into circulating red cells, it is clear that the observed increases in bone marrow mitotic activity were within the erythroid series, for EPO specifically acts upon erythroid precursors in bone marrow (see Goldwasser, 1966; General Introduction, Section 2.11). Indeed, it has been noted that during acute erythropoietic challenge prolif-

eration may even be reduced in non-erythroid bone marrow series (Hellman & Grate, 1967). The elevation of bone marrow cellular proliferation was presumably not due to a direct action of EPO upon erythroid elements since it did not occur in aparathyroid animals (Figures 33 and 34, Table 24). Thus, although it is well established that EPO does stimulate erythroblast proliferation directly, both in vivo and in vitro (Kuna, Gordon, Morse, Lane & Charipper, 1959; Matoth & Kaufmann, 1962; Blackett, 1968; Reissmann & Samorapoompichit, 1970), the increases in marrow mitotic activity observed in this study appear to be mediated by the parathyroid-dependent hypercalcaemia (compare Figures 26, 30, and 31).

The elevation of extracellular calcium ion concentration by the administration of EPO is envisaged as increasing bone marrow mitotic activity via the mechanism which was discussed in detail previously (General Introduction, Section 5.4; Figure 4). This scheme, presented by Whitfield and his colleagues (Whitfield et al., 1973c), has been derived from experiments with thymocytes maintained in vitro. However, increases in extracellular ionised calcium have also been shown to stimulate erythropoiesis in vitro (Morton, 1968; Morton et al., 1968) and evidence is presented later in this present study (Chapter 5) that the mechanism of action of the ion is probably similar to that in thymocytes. In summary, it seems likely that ionised calcium has the ability to increase the concentration of intramembranal cyclic AMP, which stimulates a membrane activation site to produce messenger substances which enter the cell and initiate the DNA synthetic and mitogenic processes within the nucleus (see Figure 4). This process could have been triggered by the elevated ionised calcium levels in the extracellular fluid which were induced by EPO (Figure 26).

The means whereby EPO interacted with the calcium homeostatic system to produce this hypercalcaemia is, however, less clear.

The increase in mobile (plasma) calcium levels produced by EPO was dependent upon the presence of the parathyroid gland (Figure 32) and presumably, therefore, upon the competence of the animal to secrete PTH. The normal stimulus for increased PTH synthesis and release is a lowering of plasma calcium concentration (c.f. McLean, 1957; Talmage, 1967b; Copp, 1969a; General Introduction, Section 4.3). Initial hypocalcaemic phases have been observed to precede the parathyroid-dependent processes of rapid liver regeneration (Rixon & Whitfield, 1972b) and post-haemorrhagic restoration of haematocrit (Perris et al., 1971; see also Chapter 3) and it was possible that such a phenomenon might occur directly after EPO administration. No hypocalcaemia was observed when plasma calcium concentrations were measured at 2, 3 and 6h after EPO injection (Figure 35, Table 23) or at 1h intervals from 0 - 6h following the hormone (Perris, unpublished observations). However, it might well be that a decrease in plasma calcium concentration was quickly obscured by a compensatory release of PTH which would quickly restore normal levels. To eliminate this possibility plasma calcium concentrations were measured 3 and 6h after EPO administration in TPTX rats (Table 23) and were found to remain steady. Thus EPO could not have a calcium-chelating or -sequestering action. It must be noted that the possibilities do remain that EPO has a very transitory inhibitory effect upon PTH-induced bone resorption or that it promotes the release of CT. It seems most unlikely, however, that the sustained PTH- dependent hypercalcaemia (Figure 35) could be stimulated by such a transitory lowering of plasma calcium levels.

If EPO were directly parathyrotrophic this would account for the sustained hypercalcaemia observed after administration of the exogenous hormone (Figures 26 - 29, 32 and 35, Table 24) and for that occurring after haemorrhage (Perris et al., 1971; see also Chapter 3); in the latter situation, circulating levels of endogenously produced EPO are supranormal (Jacobson & Goldwasser, 1958; Fried et al., 1970). An alternative mechanism for the plasma calcium-elevating ability of EPO might involve the sensitization by the hormone of some or all of PTH's target organs (bone, small intestine or kidney) to PTH itself. By this means a normal level of circulating PTH might cause a supranormal degree of bone resorption, intestinal absorption or renal tubular reabsorption of calcium. Bone resorption is the most important of these processes (General Introduction, Sections 4.2 and 4.3) and, considering also the size and protracted nature of the observed hypercalcaemia, would therefore be the most likely site of action of EPO. At the (physiological) concentrations used in the present experiments EPO clearly does not stimulate these processes directly for no significant increases in plasma calcium concentrations were noted after EPO administration to aparathyroid rats (Figure 32, Tables 23 and 24). However, in the intact animal EPO could act synergistically with PTH or exercise a permissive role for PTH-stimulated processes. It should be noted that Vitamin D metabolites and some other stimulators of bone resorption appear to share this type of action (DeLuca, 1969, 1971; Raisz, 1970; Avioli & Haddad, 1973). It is difficult to reconcile the sustained elevations of plasma calcium which have been observed after exogenous EPO and after haemorrhage (Perris et al., 1971) with the classical concepts of calcium homeostasis. Increased mobilised calcium might be expected to reduce PTH secretion (Raisz, 1963; Care et al., 1966; Sherwood et al., 1966) or stimulate CT secretion (Klein & Talmage, 1968) in most circumstances, thus leading to a return to the homeostatic norm. However, the existence of a hypercalcaemia

after EPO administration, and its importance for the stimulatory action of the hormone upon bone marrow cellular proliferation, have been firmly established (Figures 26 - 35, Tables 23 and 24).

Acid hydrolysis of the EPO preparation used in these experiments has strongly indicated that the observed effects of the extract are due to specific activities of the EPO molecule. Approximately 2.5% of the glycoprotein EPO molecule is sialic acid (Goldwasser & Kung, 1972) and removal of this moiety has been found to inactivate the hormone in vivo while not affecting its in vitro activities in bone marrow cultures (Lukowsy & Painter, 1972). This is now believed to reflect the rapid clearance of the desialated molecule from blood by the liver, in a manner analagous to that observed for sialic acid-deprived ceruloplasmin (Lukowsy & Painter, 1972; Goldwasser, Kung & Eliason, 1973). Mild acid hydrolysis at 80°C has been shown to specifically desialate the EPO molecule (Rambach et al., 1958) while heat treatment alone has little effect upon the activity of the hormone (Borsook et al., 1954; Figure 28). It is possible to argue that impurities could exist in the Step I EPO preparation which were capable of producing hypercalcaemia but which were inactivated by desialation. This seems unlikely, since injection of the plasma proteins albumin and α-globulin, which are likely to be the major contaminants of Step I preparations (Painter, Bruce & Goldwasser, 1968), did not elevate plasma calcium concentrations or bone marrow mitotic index (Figure 29).

It is well established that EPO has a number of direct actions upon erythroid precursors; these have been described previously (General Introduction, Section 2.11). In addition to promoting the release (Gordon et al., 1962) of bone marrow reticulocytes (which could not vitiate the methods used to measure mitotic index in this study since the reticulocytes are anucleate) EPO acts to promote the differentiation of stem cells (ERC) into erythroblasts and also increases

erythropoietic efficiency and rate. The primary actions of EPO are to stimulate the syntheses of RNA and stromal components and facilitate the incorporation of iron into the haemoglobin of red cell precursors (Krantz & Goldwasser, 1965; Gross & Goldwasser, 1969, 1970, 1972; Hodgson, 1970; Krantz, 1973). The stimulation of DNA synthesis and cell division is not necessary for the expression of these properties of EPO (Erslev & Hughes, 1960; Gross & Goldwasser, 1970, 1972; Ortega & Dukes, 1970). EPO does stimulate DNA synthesis and proliferation, but at a later stage than the differentiation effects mentioned above are manifest (Kuna et al., 1959; Matoth & Kaufmann, 1962; Powsner & Berman, 1967; Blackett, 1968). However, increases in erythroid cell proliferation induced by the direct action of EPO upon these elements were probably not responsible for the upsurge in marrow cell proliferation observed (Figures 30 and 31) in this study, for they were not evident in aparathyroid animals.

It could be reasoned that PTH or the elevated calcium ion concentration might have acted synergistically with, or played a permissive role for, EPO upon erythroblasts, for both ionised calcium and PTH have been demonstrated to stimulate cell division in the bone marrow both in vivo (Perris & Whitfield, 1971) and in vitro (Morton, 1968; see Chapter 5). However, injections of PTH and $CaCl_2$ have been shown to stimulate both bone marrow mitotic activity and radioiron incorporation into peripheral red blood cells in nephrectomised and polycythemic animals in which EPO levels are negligible (Jacobson et al., 1957b; Adamson & Finch, 1968; Katz et al., 1968). Furthermore, the ambient concentrations of PTH which are likely to have been present in the extracellular fluid of the animals used in the present study are much lower than those which stimulated bone marrow cell proliferation in vivo or in vitro (Perris & Whitfield, 1971; Chapter 5). In contrast, increases in plasma total and ionised calcium concentrations of some 10%, as observed in the current experiments (Figure 26), have been observed to elicit

large (30 - 80%) increases in bone marrow mitosis and radioiron incorporation into circulating red blood cells (Perris et al., 1967; Rixon, 1968; Perris & Whitfield, 1971). These values correspond well with the increases in bone marrow mitosis (35 - 70%) produced by 10u/100g body weight of Step I EPO (Figures 30 and 31; Table 24) in intact animals.

It therefore seems likely that the mitogenic effect of elevated extracellular ionised calcium concentrations could amplify the small stimulatory effects of EPO upon bone marrow cell proliferation. This could be envisaged to occur in a number of ways:

(i) CFU (pluripotential stem cells) are a population of which less than 10% are normally actively cycling (Becker et al., 1965; Lajtha et al. 1969). Recruitment of further cells into the cycle would not increase measurable mitotic activity per se, since the numbers of CFU in the bone marrow are only a very small percentage of the total marrow cell population. However, recruitment would provide a larger base of cells from which ERCs (unipotential stem cells) could be derived. Note that since the ERCs are all in cycle, some 70% of which is spent in DNA synthesis (Lajtha et al.1969), it is unlikely that calcium could significantly affect the proliferation kinetics of this committed population, for as discussed previously (General Methodology, Section 6.3) it is probable that the duration of the S phase remains relatively stable in most mammalian cell types. However, EPO itself has been postulated to influence the cytokinetics of the ERC population (Reissmann & Samorapoompichit, 1970).

(ii) Rat proerythroblasts normally undergo 7 divisions before reaching the mitotically incompetent normoblast stage (Tarbutt & Blackett, 1968). The generation time (i.e. the interval between mitoses) of the various erythroblasts is in the range 12 - 15h (Alpen & Cranmore, 1959).

Elevation of extracellular calcium could therefore stimulate erythroblast mitosis and thereby shorten marrow transit time.

(iii) If calcium stimulated division in cells which had undergone 7 division stages, or in cells which, although still mitotically competent, had "skipped" one or more division phases (Alpen & Cranmore, 1959), the "yield" of erythrocytes would be increased. Such an "extra division" capacity has been suggested for EPO itself (Blackett, 1968).

Regardless of the mechanism responsible for the action of EPO-induced hypercalcaemia upon bone marrow cellular proliferation, it is clear that the importance of the parathyroid gland in the control of erythropoiesis has been further emphasized by the current observations (Figures 26 - 35, Tables 23 and 24). If endogenous EPO acts similarly to the exogenous preparation, one can readily explain a number of experiments which have demonstrated increases in plasma calcium concentration in heightened erythropoietic circumstances. For example, after cobaltous chloride treatment, haemorrhage, or during pregnancy, situations in which circulating EPO levels are elevated (Contopoulos, Van Dyke & Simpson, 1956; Jacobson & Goldwasser, 1958; Fried et al., 1970), rat plasma calcium concentrations and bone marrow mitosis increase in parallel (Perris, 1971; Perris et al., 1971). These observations may also have relevance in man, for during the heightened erythropoiesis which presumably counteracts the inadequate circulation and tissue oxygen supply of children with certain cardiac defects plasma ionised calcium concentrations are elevated (Putman, 1972). Although at present no evidence has been obtained which relates abnormalities in erythropoiesis to clinical derangements in calcium homeostasis this may simply be because the possibility of such relationships has not previously been established. During a long-term condition such as hypoparathyroidism it is likely that any reduction in erythro-

poietic activity would be partially compensated for by the other arm of the erythropoietic control system, i.e. by increased elaboration and release of EPO which would act directly upon bone marrow elements. These possibilities remain to be investigated.

Table 24 overleaf.

	Plasma Calcium Concentration (mg/100 ml)				Bone Marrow Mitotic Activity			
Treatment	Total	P	Ionised	P	% Nucleated cells in metaphase 6 h after Colcemid	P	Mitotic Index	P
Normal + saline	10.4 ± 0.1 (28)	<0.001	5.2 ± 0.05 (10)	<0.001	14.5 ± 0.6 (20)	<0.001	1.2 ± 0.1 (14)	<0.001
Normal + EPO	11.5 ± 0.1 (22)		5.8 ± 0.05 (10)		19.7 ± 0.9 (20)		1.8 ± 0.1 (8)	
TPTX + saline	6.2 ± 0.2 (20)	<0.01	2.2 ± 0.15 (5)	NS	9.7 ± 0.6 (5)	NS	0.95± 0.1 (5)	NS
TPTX + EPO	6.6 ± 0.2 (18)		2.3 ± 0.30 (4)		11.5 ± 0.6 (5)		1.05± 0.2 (5)	
PTX + saline	6.4 ± 0.2 (9)	NS	2.9 ± 0.20 (3)	NS	–	–	0.9 ± 0.1 (3)	NS
PTX + EPO	6.0 ± 0.3 (9)		2.5 ± 0.25 (3)		–		0.9 ± 0.1 (3)	

Table 24: Summary of the effects of exogenous EPO upon plasma calcium concentrations and bone marrow mitotic activity in normal and aparathyroid rats. Step I EPO (IOU/100 g body wt.) was injected s.c. (protocol in Methods) into normal, TPTX and PTX animals. Test parameters were measured 24 h later.
Values are means ± S.E.M. Numbers of animals given in parentheses.

Summary.

Injection of EPO has been shown to significantly increase the concentrations of both total and ionised calcium in the plasma of intact rats. This property was probably a specific action of the EPO molecule.

Bone marrow cellular proliferation increased in parallel with the EPO-induced hypercalcaemia.

Both the calcium elevation and the surge of bone marrow mitotic activity after EPO were abolished by prior extirpation of the parathyroid glands.

Since no hypocalcaemic phase was evident after EPO administration to intact or TPTX rats it was postulated that EPO was probably either parathyrotrophic or exerted a permissive action for the effects of PTH upon its classic target organs. EPO itself did not significantly enhance bone resorption, intestinal calcium absorption or renal tubular reabsorption of calcium.

It was concluded that the stimulation of bone marrow mitosis by EPO was via the elevation of plasma ionised calcium concentration and that these observations substantiated previous indications of the importance of the calcium homeostatic system in the maintenance of normal erythropoiesis and the capacity to adjust red cell numbers in response to hypoxic stimuli.

CHAPTER 3.

Hormonal co-operation in the control of the bone marrow proliferative response to haemorrhage.

Introduction.

The observation that exogenous administration of erythropoietin (EPO), the hormone which regulates red cell production, increased bone marrow mitotic activity via the elevation of mobile body calcium (Chapter 2) suggested that the endogenously produced hormone might share a similar mechanism. A number of erythropoietic stimuli and inhibitors have been discussed (General Indtroduction, Sections 2.5 and 2.12) which all affect EPO production by causing shifts in the tissue oxygen supply/demand ratio. Some of these are discussed in the following Chapter (4). The studies reported in the present Chapter have been performed to elucidate the nature of the erythropoietic response to loss of circulating red cells by haemorrhage.

Removal of blood from rats causes a reduction in the oxygen-carrying capacity of the blood and therefore decreases the oxygen supply/demand ratio; this hypoxia stimulates the production of EPO (Fried et al., 1957; Jacobson & Goldwasser, 1958; Fried et al., 1970; General Introduction, Sections 2.4, 2.5 and 2.7) which acts to increase red cell production (General Introduction, Section 2.11). Thus haemorrhage is a suitable stimulus for the production of endogenous EPO. Furthermore, increases in bone marrow mitotic activity which paralleled increases in the calcium concentration in the circulation have been demonstrated to follow severe haemorrhage in rats (Perris et al., 1971), which suggested that elevated circulating endogenous EPO levels might indeed be associated with the elevation of plasma calcium concentration.

The sudden withdrawal of large proportions of mammalian blood volumes (25 - 40%) induces a number of compensatory mechanisms which are primarily concerned with the maximisation of the efficiency of oxygen transport during the period of red cell deficiency. In addition to the secretion of EPO, a variety of hormones are released which act

co-operatively to maintain arterial blood pressure and thus the blood flow through peripheral vessels. For example, the release of medullary catecholamines (Walker, Zileli, Reuter, Schoemaker, Friend & Moore, 1959) complements the increase in sympathetic vasoconstrictor activity triggered by the baroreceptors located in the carotid sinus and aorta (Heymans & Neil, 1958). Anti-diuretic hormone (ADH), renin/angiotensin, adrenocorticotrophic hormone (ACTH), cortisol and aldosterone are also secreted after haemorrhage (Ginsburg & Heller, 1953; Sydnor & Sayers, 1954; Davis, 1961, 1962; Eilers & Peterson, 1964; Muller, Manning, Moret & Mégevand, 1963; Scornik & Paladini, 1964; Lodge, Lowe & Vane, 1966; Errington & Rocha e Silva, 1971; Johnson, Davis, Brown, Baumber & Waid, 1971; Cousineau, Gagnon & Sirois, 1973) and act to retain body fluid, restore blood volume and re-establish fluid/salt balance. Thus any of these hormones might, if mitogenic, enhance bone marrow cellular proliferation in the period immediately following haemorrhage, when their circulating levels are maximal.

Rapid removal of 30 - 40% of the blood volume of a rat has been demonstrated to induce a protracted parathyroid-dependent hypercalcaemia which commenced at 24h and persisted until the haematocrit returned to normal (Perris et al. 1971). This elevated extracellular calcium concentration stimulated bone marrow mitosis and was essential for the rapid restoration of haematocrit. The observation that exogenous EPO stimulated bone marrow mitotic activity via the elevation of plasma calcium concentration (Chapter 2) suggested that release of endogenous EPO could have produced the parallel increases in mobile calcium and bone marrow cellular proliferation reported after haemorrhage (Perris et al., 1971). However, these workers also noted a period of hypocalcaemia which developed within 4h of bleeding and persisted for a further 16h. This lowering of plasma calcium concentration might be expected to reduce bone marrow mitotic activity in a manner analagous

to removal of the parathyroid glands (Rixon, 1968; Perris & Whitfield, 1971; Rixon & Whitfield, 1972a; Chapters 1 and 2). Since this would presumably be physiologically disadvantageous, it seemed pertinent to investigate the possibility of the existence of mitogenic influences which were essentially calcium independent and which might therefore oppose the hypoplastic trend. In addition, the aetiology of the hypocalcaemic phase itself has not been definitely elucidated. Preliminary experiments had indicated that the time scale of the responses of bone marrow cellular proliferation and of the calcium homeostatic system to haemorrhage was different in normal rats to that observed by Perris et al. (1971) in specific pathogen free animals.

The studies reported in this chapter were therefore undertaken to:

(i) Examine the time scale of changes in calcium homeostasis and bone marrow cellular proliferation after haemorrhage in normal Wistar rats.

(ii) Investigate the factors responsible for both the hypocalcaemic phase and the subsequent protracted hypercalcaemia.

(iii) Measure bone marrow mitotic activity during the hypocalcaemic phase and determine whether these observations could be explained simply in terms of the interaction of EPO and the calcium homeostatic system.

(iv) Investigate the mitogenicity of several hormones which are known to be secreted in supranormal amounts after haemorrhage.

Table 25 overleaf.

Time after Sham-Bleeding	Plasma Calcium Concentration (mg/100 ml)				Bone Marrow Mitotic Index	
	Total		Ionised			
	Sham-Bled	Intact	Sham-Bled	Intact	Sham-Bled	Intact
2	10.75 ± 0.1 (4)	10.7 ± 0.1 (4)	5.60 ± 0.10 (4)	5.45 ± 0.15 (4)	1.5 ± 0.2 (4)	1.4 ± 0.2 (4)
4	10.8 ± 0.1 (4)	10.9 ± 0.2 (4)	5.60 ± 0.10 (4)	5.60 ± 0.05 (4)	1.4 ± 0.1 (4)	1.6 ± 0.2 (4)
24	10.4 ± 0.1 (5)	10.3 ± 0.1 (4)	5.50 ± 0.05 (5)	5.20 ± 0.05 (4)	1.3 ± 0.1 (5)	1.45± 0.1 (4)
48	10.2 ± 0.1 (5)	10.0 ± 0.2 (5)	4.90 ± 0.10 (5)	5.00 ± 0.05 (5)	1.2 ± 0.1 (5)	1.3 ± 0.1 (5)

Table 25: Absence of effect of sham-bleeding upon test parameters. Rats were sham-bled (see General Methodology, Section 4.3) at 0h and sacrificed at the times shown. Normal, untreated animals were killed at the same times. There were no significant differences in plasma total and ionised calcium or in bone marrow mitotic index between sham-bled and intact (untreated) animals after any of the intervals studied. Numbers of animals given in parentheses.

Methods.

In the majority of experiments bone marrow mitotic activity and plasma calcium concentration were measured at various intervals after the removal of blood from male rats. These parameters were also measured after the administration of hormones in concentrations equivalent to those endogenously secreted after haemorrhage (see Discussion).

Blood (2.0 - 2.5ml/100g body weight) was withdrawn from animals by cardiac puncture in heparinised syringes (see General Methodology, Sections 4.3, 5.1 and 5.3). Plasma was prepared and stored as before (Methodology, Section 5.1). The concentrations of plasma total and ionised calcium inorganic phosphate and protein were measured as described previously (Methodology, Sections 5.1, 5.2, 5.3, 5.5 and 5.6). Control animals were sham-bled (Methodology, Section 4.3). This sham procedure did not significantly affect any of the parameters under investigation (Table 25), indicating that stress, cardiac damage or exposure to ether were not capable of obscuring the true nature of changes in blood composition or bone marrow mitotic activity after haemorrhage. Furthermore, in some experiments "internal controls" could be exployed, i.e. the concentrations of blood constituents after various short post-haemorrhage periods could be compared with those in the blood which had been removed initially. Note that in these internal control situations it was particularly important that experiments were terminated before the onset of the sharp diurnal decreases in plasma calcium concentration which have been observed between 16.00 and 20.00 in conditioned rats (Results, Chapter 1). Small numbers of sham-bled controls were employed in all experiments using internal controls to ensure that diurnal changes had not occurred in the non-conditioned experimental animals.

As an indication of the magnitude of erythropoietic demand haematocrit and sometimes blood haemoglobin were measured (see Supplement 1,

immediately following the present section, for techniques) at various times after haemorrhage. In some cases, white (nucleated) blood cell counts were performed with the Coulter Counter ZB1 (Methodology, Section 8). Bone marrow mitotic activity was assessed both by the determination of mitotic index and with the use of the metaphase-arresting agent Colcemid (Methodology, Section 6).

In some experiments the blood removed from the heart was immediately (within 20 - 40 seconds) replaced by intracardial reinfusion of homologous donor whole blood, plasma or blood containing red cells saturated with carbon monoxide (CO). In all cases the reinfused fluid contained low (approximately 2 i.u./ml) concentrations of heparin and was at 37°C. Particular care was required to prevent reinfusion of air bubbles, which was invariably fatal. The preparation of the reinfusates is described in the second supplement to this Methods section.

Plasma calcium concentration and bone marrow mitotic activity were measured in intact animals 4h after the injection of anti-diuretic hormone (ADH - "Pitressin", Parke-Davis Co.), aldosterone ("Aldocorten", CIBA Ltd.), angiotensin II amide (CIBA, Ltd.) or (1-deamino-8-D-arginine) - vasopressin (DDAVP - "Desurin", Reckitt and Colman, Ltd.). ADH (6mU/100g body weight) was administered as a single intracardial injection in 0.1ml 0.9% saline. DDAVP, a synthetic analogue of ADH with potent antidiuretic - but little vasopressor - activity (Zaoral, Kolc & Sorm, 1967; Vavra, Machova, Holecek, Cort, Zaoral & Sorm, 1968) was administered by the same route; the doses were 75 or 100pg/100g body weight. Aldosterone and angiotensin were given as divided doses. Aldosterone was injected i.p. (75ng/100g body weight) and intramuscularly (1.25µg/100g body weight), both doses being administered in 0.1ml 0.9% saline. Angiotensin was injected i.m. (75ng/100g body weight) and s.c. (40ng/100g body weight) in 0.1ml saline. The rationale for these doses and routes of administration is considered later (Discussion).

To determine whether ADH, aldosterone, angiotensin II amide and EPO could stimulate bone marrow mitosis directly they were studied in an _in vitro_ bone marrow cell culture system (see Methodology, Sections 7.3 and 7.4 for detailed description and discussion of this technique).

The possible contributions of PTH, adrenal hormones and pituitary hormones to the compensatory mechanisms operating after haemorrhage were assessed by bleeding (2.0 - 2.5ml blood/100g body weight) parathyroidectomised (PTX), adrenalectomised (ADX) and hypophysectomised (HX) rats. Plasma calcium concentration and bone marrow mitotic activity were measured 4h later. Animals were parathyroidectomised as before (Methodology, Section 4.2) and adrenalectomised as described in Supplement 3. HX rats were obtained from a commercial source (Carworth Europe) and bled 5 days after they had undergone surgery. Both ADX and HX animals were supplied with drinking water containing 0.9% NaCl and 1% glucose after removal of the glands. NaCl was supplied continuously until sacrifice to oppose the urinary loss of sodium which occurs in the absence of aldosterone secretion from the adrenals; aldosterone secretion may also be influenced by adrenocorticotrophic hormone (ACTH) from the anterior pituitary. Glucose was supplied only for the first 2 days after the surgery to lessen the severity of post-operative trauma.

Supplement 1: Measurement of haematocrit and haemoglobin.

Haematocrit (the percentage volume of whole blood occupied by red blood cells) was measured by the micro-haematocrit method in heparinised capillary tubes (Harshaw Chemicals). The tubes were contrifuged for 2.5 minutes in a Hawksley micro-haematocrit constant speed centrifuge. The "Haematocrit Reader" supplied by Hawksley was found to be inaccurate and haematocrit was therefore calculated from direct measurements of the lengths of the whole blood and red cell columns in the capillary tubes.

Table 26 overleaf.

Sample	Haematocrit (packed red cell volume as % whole blood volume)	
	Mean ± s.e.m.	Range
1	40.5 ± 0.5 (4)	39.5 - 42
2	41 ± 0.5 (4)	40.5 - 42.5
3	43 ± 0.5 (4)	41 - 44
4	42 ± 0.5 (4)	41 - 44
5	42 ± 0.0 (6)	40.5 - 42.5

Table 26: Repeatability of the micro-method for measurement of haematocrit. Numbers of determinations given in parentheses.

Tables 27 and 28, overleaf.

Sample	Haemoglobin concentration (g/100ml)	
	Mean	Range
1	12.8 ± 0.0	12.8 - 12.9
2	15.1 ± 0.1	15.0 - 15.2
3	17.0 ± 0.1	17.0 - 17.2
4	14.6 ± 0.1	14.6 - 14.8
5	15.2 ± 0.0	15.1 - 15.2
6	13.8 ± 0.0	-

Table 27: repeatability of the cynanmethaemoglobin method for haemoglobin. Values in column 2 are means ± s.e.m. from 4 determinations in each case.

Sample	Haemoglobin concentration (g/100ml)	
	Mean	Range
1	14.2 ± 0.1	14.0 - 14.3
2	12.6 ± 0.2	12.3 - 12.8
3	16.2 ± 0.1	16.1 - 16.3
4	15.7 ± 0.1	15.5 - 15.8
5	13.6 ± 0.1	13.5 - 13.8

Table 28: reproducibility of the cyanmethaemoglobin method for haemoglobin. The haemoglobin content of 5 blood samples was determined in quadruplicate. Values in column 2 are means ± s.e.m.

The reproducibility of the method was found to be satisfactory (Table 26). It should be noted that the haematocrit gives at best only an indication of circulating red cell status since changes in plasma volume affect haematocrit without necessarily altering the tissue oxygen demand/supply ratio (i.e. the erythropoietic demand - General Introduction, Sections 2.5 and 2.7). This criticism may also be levelled at haemoglobin measurements made independently of blood volume determinations.

Haemoglobin in blood was measured by the cyanmethaemoglobin method in an EEL haemoglobinometer (Evans Electrosolenium Ltd.). 20µl of blood was mixed with 4ml alkaline Drabkin reagent and, after standing 10 minutes for colour development, was read in comparison with 3 and 18g haemoglobin/100ml cyanmethaemoglobin standards. The repeatability and reproducibility of the method were satisfactory (Tables 27 and 28). From this data, and considering also the minimum change which was detectable on the haemoglobinometer scale (± 0.05/100ml), the limit of precision of the method was assessed to be ± 0.1g haemoglobin/100ml.

Supplement 2: Preparation of Reinfusates.

Whole blood was drawn from healthy male donors in heparinised syringes and pooled in a vessel containing a small volume of heparinised saline. The final concentration of the anticoagulant was approximately 2i.u./ml, i.e. twice the normal level (see General Methodology, Section 5.3 and Figure 8).

Homologous plasma was prepared by centrifuging the whole blood at 500 x g for 10 minutes and drawing off the plasma supernatant. Samples showing evidence of haemolysis were discarded.

In one study, red cells were saturated with carbon monoxide (CO) prior to reinfusion to produce a deficiency in blood oxygen-carrying

capacity without reducing blood viscosity and flow dynamics. To prevent the possible dissolution of CO in the plasma during this procedure, whole blood was first centrifuged at 500 x g for 10 minutes. Plasma was drawn off and retained. The red cells were resuspended in an equal volume of 0.9% saline and then slowly bubbled with CO in a vessel equipped with a magnetic stirrer and maintained at 37°C. When CO saturation was complete (see below) the red cell suspension was re-centrifuged, the saline supernatant was discarded, and the red cells were then re-suspended in the original plasma. Measurements of haemoglobin, haematocrit and visual monitoring of the colour of the saline supernatant were employed to ensure that badly haemolysed samples were not reinfused. This procedure ensured that dissolved CO, which might have become transferred to red cell haemoglobin in the recipient's circulation, was not present in the blood reinfusate. Control samples were treated similarly but were not bubbled with a gas.

The saturation of haemoglobin by CO was measured by the method of Klendshoj, Feldstein & Sprague (1950). The principle is as follows: if a pigment, A, in solution has an optical density (O.D.) Dx at one light wavelength (x) and Dy at another (y) and a pigment B has optical densities dx and dy at the same two wavelengths, then in a mixture of A and B the numerical ratio of its optical densities at x and y has been shown to lie between the quotients Dx/Dy and dx/dy. Thus a relationship may be derived between the composition of the mixture and its O.D. quotient at two suitable wavelengths of light. In this procedure the pigments are carboxyhaemoglobin and reduced haemoglobin. A straight line relationship has been established between the composition of mixtures of the two pigments in blood and the O.D. ratio for measurements at 480 and 555mμ (Klendshoj _et al._, 1950). Oxyhaemoglobin is reduced with sodium hydrosulphite (sodium dithionate) which does not affect the binding of CO to haemoglobin. Blood samples are diluted

1/100 in a lysing agent (0.4% ammonia) prior to reading.

The method was used only as a rough estimation of CO saturation of red cell haemoglobin and was therefore not examined in depth. CO was bubbled through pooled blood samples until the D_{555}/D_{480} quotient was maximal, plus a further 5 minutes. At the low perfusion rate which was employed (to avoid haemolysis) 40ml of red cells suspended in saline were completely saturated with CO in 30 - 35 minutes.

The preparative procedures for the three reinfusates (viz. whole blood, homologous plasma, and CO-treated blood) were conducted, as far as practicable, in anaerobic conditions (i.e. under oil coverage) to prevent loss of CO_2 and the consequent changes in blood pH and plasma ionised calcium concentration. This was not possible in some stages of the preparation of CO saturated blood; pH was not re-adjusted prior to reinfusion. All reinfusates were at 37°C when administered. At the end of the experiments involving cardiac infusion the thoracic cavity was examined for signs of leakage from the heart. This was observed on three occasions and these animals were discarded.

Supplement 3: Adrenalectomy.

Animals weighing 140-160g were adrenalectomised (ADX) through lateral abdominal incisions under ether anaesthesia. Adrenal blood vessels were closed by pressure and the adrenal glands were then removed by blunt dissection. The ADX rats were supplied with glucose and saline in the drinking water as described earlier (Methods, this Chapter). See General Methodology, Section 4.1 for a general description of surgical technique.

Expression of Results.

In studies conducted at intervals over long periods of time, as were those reported in this Chapter, seasonal variations in many experimental parameters were observed . Plotting graphically the absolute values of these parameters observed in control and experimental animals often produced misleading impressions of the trends which were occurring. For example, a number of measurements made in groups of untreated animals at different times of the year are tabulated below (Table 29):

Date	No. of Animals	Plasma Calcium Concentration (mg/100ml)		Bone Marrow Mitotic Index	Haematocrit
		Total	Ionised		
2.2	5	10.4 ±0.1	-	1.8 ± 0.1	43 ± 0.5
3.3	5	10.5 ±0.1	5.45±0.05	-	-
12.5	5	9.9 ±0.4	4.60±0.10	0.9 ± 0.1	-
5.6	5	10.25±0.2	5.15±0.20	1.25± 0.0	-
1.7	5	10.2 ±0.2	5.00±0.10	1.25± 0.1	41 ± 1.5
16.7	8	10.05±0.1	5.15±0.05	1.1 ± 0.1	-
11.8	5	10.4 ±0.1	-	1.8 ± 0.2	43 ± 0.5
13.9	5	9.5 ±0.1	-	1.0 ± 0.1	-
21.9	5	10.0 ±0.1	-	1.35± 0.3	-

Table 29: Seasonal variations in test parameters in normal rats.

The few selected results presented in the table clearly demonstrate that fluctuations in control parameters exist. These could be due to sampling variation or to seasonal changes, but in either eventuality pictorial presentations of results may not truly represent the magnitude and pattern of post-haemorrhagic changes if absolute values of parameters in experimental and control animals are compared. Thus in some Figures in this Chapter, variations in test parameters are expressed as absolute or percentage changes from control values. Thus the s.e.m. of points

in such figures will represent the standard error of the mean increase from the control value, whether this increase is expressed in absolute terms or as a percentage. Points which represent values which are significantly different from controls ($P < 0.05 \longrightarrow P < 0.001$) are given in red. Other points are black.

Days After Haemorrhage or Sham-Bleeding	Haematocrit (%)				
	Sham-bled	Bled	△	P	% Decrease
1	39.5 ± 1.0 (5)	27.5 ± 1.0 (5)	-12.0	<0.001	30.4 ± 2.5
1.3	39.5 ± 1.5 (3)	28.5 ± 2.5 (4)	-11.0	<0.001	27.9 ± 6.3
2	40.5 ± 0.5 (9)	30.0 ± 1.0(11)	-10.5	<0.001	26.0 ± 2.5
3	41.5 ± 0.5 (7)	32.0 ± 1.5 (9)	-9.5	<0.001	22.9 ± 3.6
4	43.0 ± 1.0 (5)	36.5 ± 0.5 (6)	-6.5	<0.001	15.1 ± 1.2
6	41.0 ± 1.5 (6)	38.0 ± 1.0 (6)	-3.0	=0.05	7.3 ± 2.4
7	41.0 ± 1.5 (6)	37.5 ± 1.5 (5)	-3.5	<0.05	8.5 ± 3.6
8	42.0 ± 0.5 (5)	40.0 ± 1.5 (4)	-2.0	>0.05	4.8 ± 3.6
9	43.0 ± 0.5 (5)	41.0 ± 1.0 (5)	-2.0	>0.05	4.6 ± 2.3

Data plotted as Figure 36A/B overleaf. Number of animals indicated in parentheses.

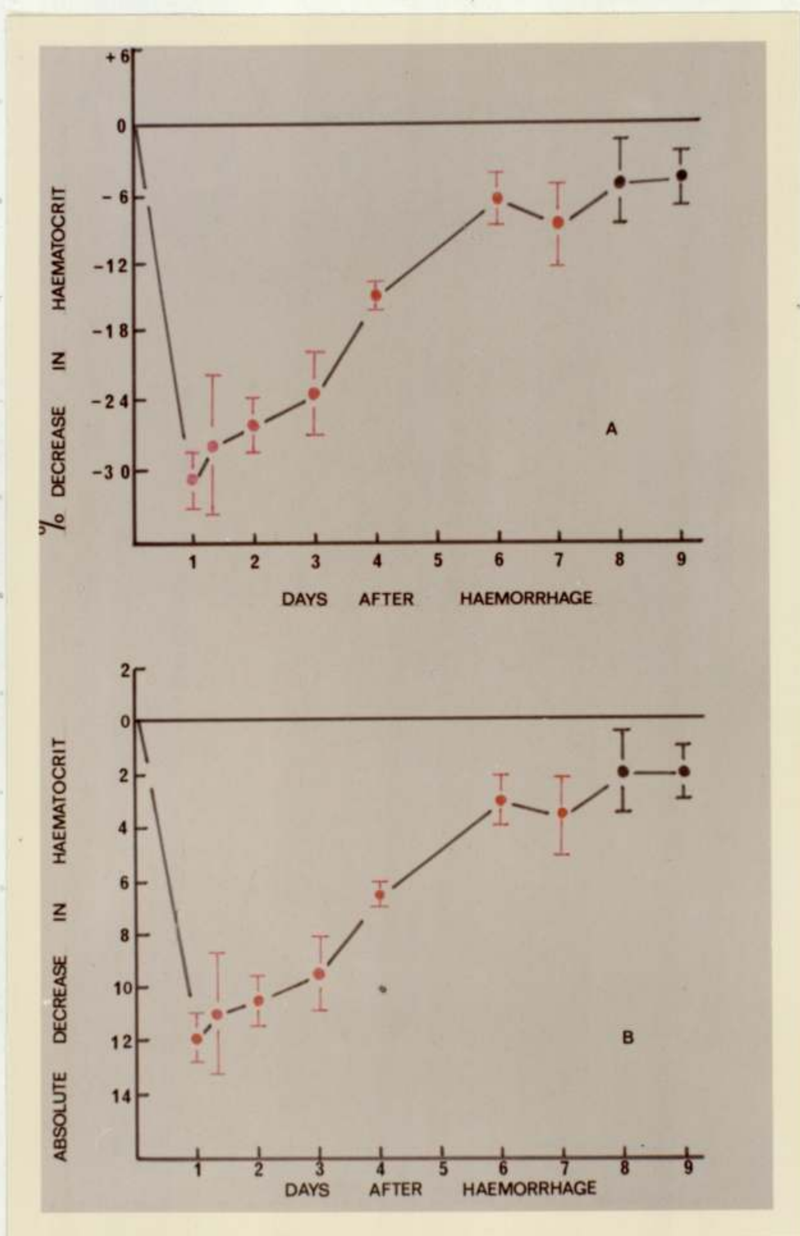


Figure 36: Haematocrit following haemorrhage in rats. Blood (2.0-2.5ml/100g body weight) was removed by cardiac puncture on day 0. The decreases in haematocrit are expressed as % of the control values (A) and as absolute values (B). Each point is mean ± s.e.m. derived from the results from 4-11 bled animals and 3-9 sham-bled controls. Points in red represent significant decreases (P between <0.05 and <0.001).

Days After Haemorrhage or Sham-bleeding	Bone Marrow Mitotic Index				
	Sham-Bled	Bled	△	P	% Increase
1	1.3 ± 0.2 (6)	1.7 ± 0.2 (4)	0.4	>0.1	30.8 ± 15.4
2	1.3 ± 0.1 (5)	1.9 ± 0.1 (6)	0.6	<0.01	46.2 ± 7.7
3	1.1 ± 0.1 (6)	1.8 ± 0.1(10)	0.7	<0.001	63.7 ± 9.1
4	1.3 ± 0.1 (5)	2.2 ± 0.2 (6)	0.9	<0.05	69.3 ± 15.4
6	1.25 ± 0.1 (6)	1.9 ± 0.1 (5)	0.65	<0.001	52.0 ± 7.1
7	1.25 ± 0.1 (6)	1.8 ± 0.1 (5)	0.55	<0.001	44.1 ± 8.0
8	1.45 ± 0.1 (6)	2.1 ± 0.1 (4)	0.65	<0.001	44.8 ± 8.1
9	1.8 ± 0.1 (5)	1.9 ± 0.2 (5)	0.1	>0.5	5.6 ± 11.2

Data plotted as Figures 37A/B overleaf. Numbers of animals given in parentheses.

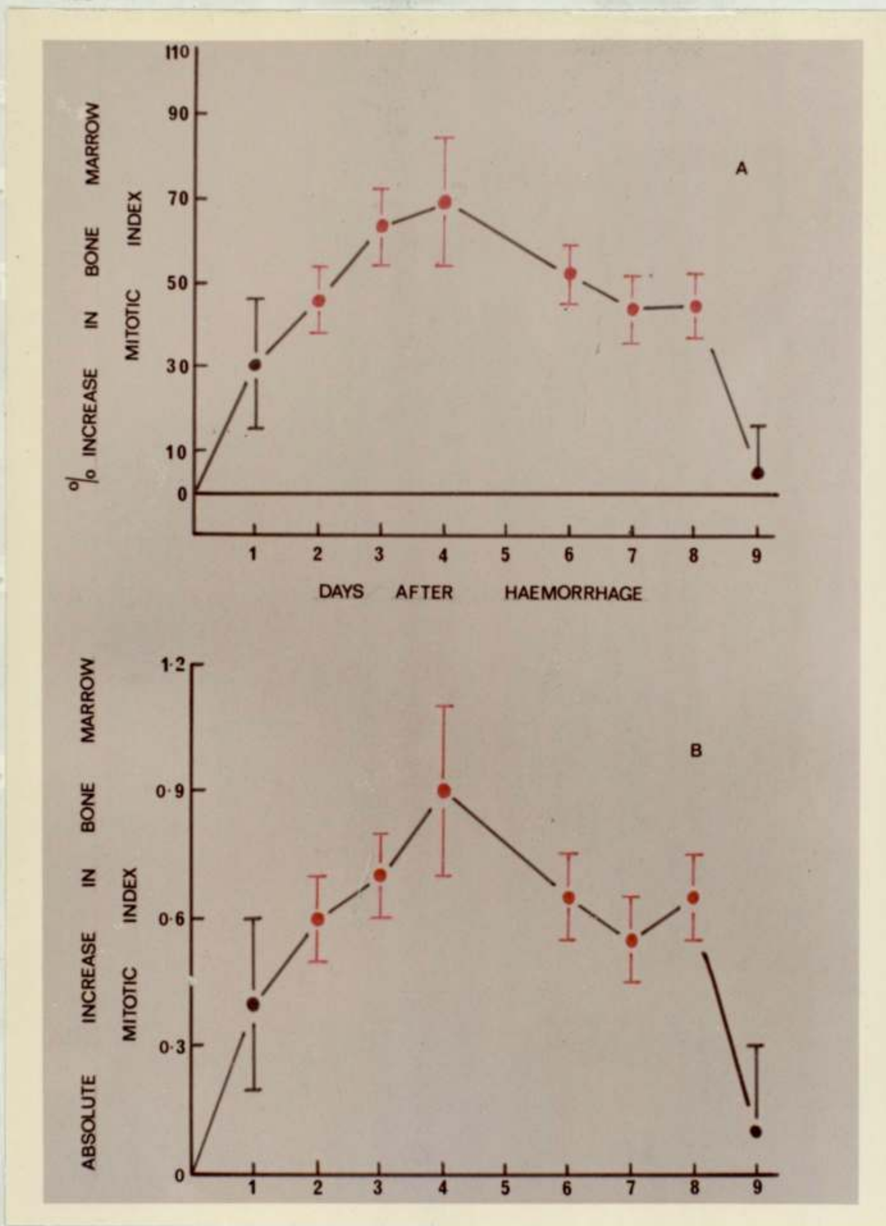


Figure 37: Bone marrow mitotic index following haemorrhage in rats. Blood (2.0 - 2.5ml/100g body weight) was removed by cardiac puncture on day 0. The increases in mitotic index are expressed as % of the control values (A) or as absolute values (B). Each point is mean ± s.e.m. derived from the results from 4-10 bled animals and 5-6 sham-bled controls. Points in red represent significant increases (P between <0.05 and <0.001).

Days After Haemorrhage or Sham-Bleeding	Plasma Total Calcium Concentration (mg %)				
	Sham-Bled	Bled		P	% Change
1	10.35 ± 0.1 (17)	10.25 ± 0.2 (9)	-0.10	>0.5	1.0 ± 2.0
2	10.1 ± 0.1 (9)	10.4 ± 0.1 (11)	+0.30	<0.05	3.0 ± 1.0
3	9.8 ± 0.1 (7)	10.2 ± 0.1 (10)	+0.40	<0.01	4.1 ± 1.0
4	10.05 ± 0.1 (5)	10.6 ± 0.1 (6)	+0.55	<0.01	5.5 ± 1.0
6	10.2 ± 0.1 (6)	10.5 ± 0.1 (6)	+0.30	=0.05	2.9 ± 1.0
7	10.2 ± 0.1 (6)	10.65 ± 0.1 (5)	+0.45	<0.05	4.4 ± 1.0
8	9.9 ± 0.0 (5)	10.0 ± 0.2 (4)	+0.10	>0.5	1.0 ± 2.0
9	10.4 ± 0.1 (5)	10.4 ± 0.2 (5)	0	-	0

Data plotted as Figure 38A/B overleaf. Numbers of animals indicated in parentheses.

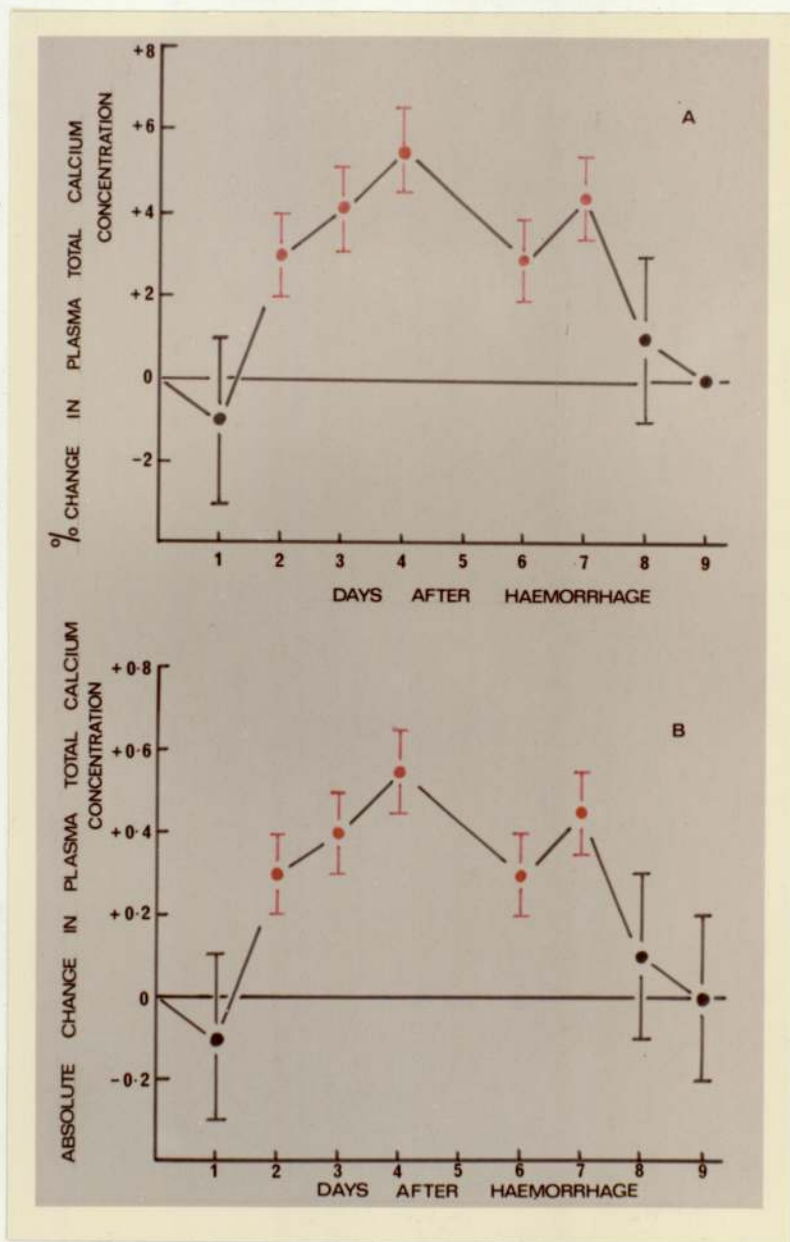


Figure 38: Plasma total calcium concentration following haemorrhage in rats. Blood (2.0 - 2.5ml/100g body weight) was removed by cardiac puncture on day 0. Changes in plasma calcium concentration are expressed as % of the control values (A) or as absolute values (B). Each point is mean ± s.e.m. derived from the results from 4-11 bled animals and 5-17 sham-bled controls. Points in red represent significant changes (P between <0.05 and <0.01).

T	Plasma Calcium Concentration (mg/100 ml							
	Total				Ionised			
	Sham-Bled	Bled	P	% change	Sham-bled	Bled	P	% change
2	10.8 ± 0.1 (13)	10.1 ± 0.3 (4)	-0.7 <0.001	6.5 ± 2.8	5.55 ± 0.1 (9)	4.95 ± 0.15 (4)	-0.6 <0.001	10.8 ± 2.7
4	10.6 ± 0.1 (28)	9.7 ± 0.2(14)	-0.9 <0.001	8.5 ± 1.9	5.50 ± 0.05(18)	5.35 ± 0.05(14)	-0.15 >0.05	2.7 ± 1.0
6	10.2 ± 0.2 (4)	9.5 ± 0.1 (5)	-0.7 <0.05	6.9 ± 1.0	5.00 ± 0.05 (4)	4.8 ± 0.10 (5)	-0.2 >0.05	4.0 ± 2.0
18	10.1 ± 0.1 (5)	9.65± 0.2 (5)	-0.45 <0.05	4.5 ± 2.0	5.25 ± 0.2 (5)	5.1 ± 0.10 (5)	-0.15>0.1	2.9 ± 2.0
20	10.2 ± 0.1 (4)	9.9 ± 0.2 (5)	-0.3 >0.1	2.9 ± 2.0	5.10 ± 0.1 (4)	4.8 ± 0.05 (5)	-0.3 <0.05	5.9 ± 1.0
24	10.35± 0.1 (17)	10.25± 0.2 (9)	-0.1 >0.5	1.0 ± 2.0	5.20 ± 0.1 (5)	5.15 ± 0.15 (5)	-0.05>0.5	1.0 ± 3.0
28	10.05± 0.1 (8)	10.25± 0.0 (8)	+0.2 >0.05	2.0 ± 0.0	5.15 ± 0.05 (8)	5.4 ± 0.05 (8)	+0.25<0.05	4.9 ± 1.0
32	10.4 ± 0.1 (5)	9.9 ± 0.2 (6)	-0.5 >0.05	4.8 ± 1.9	-	-	-	- -
48	10.1 ± 0.1 (9)	10.4 ± 0.1(11)	+0.3 <0.05	3.0 ± 1.0	5.10 ± 0.05 (5)	5.35 ± 0.05 (6)	+0.25<0.05	4.9 ± 1.0

Data plotted as Figure 39 A/B overleaf. Numbers of animals indicated in parentheses

T = hours after haemorrhage or sham-bleeding

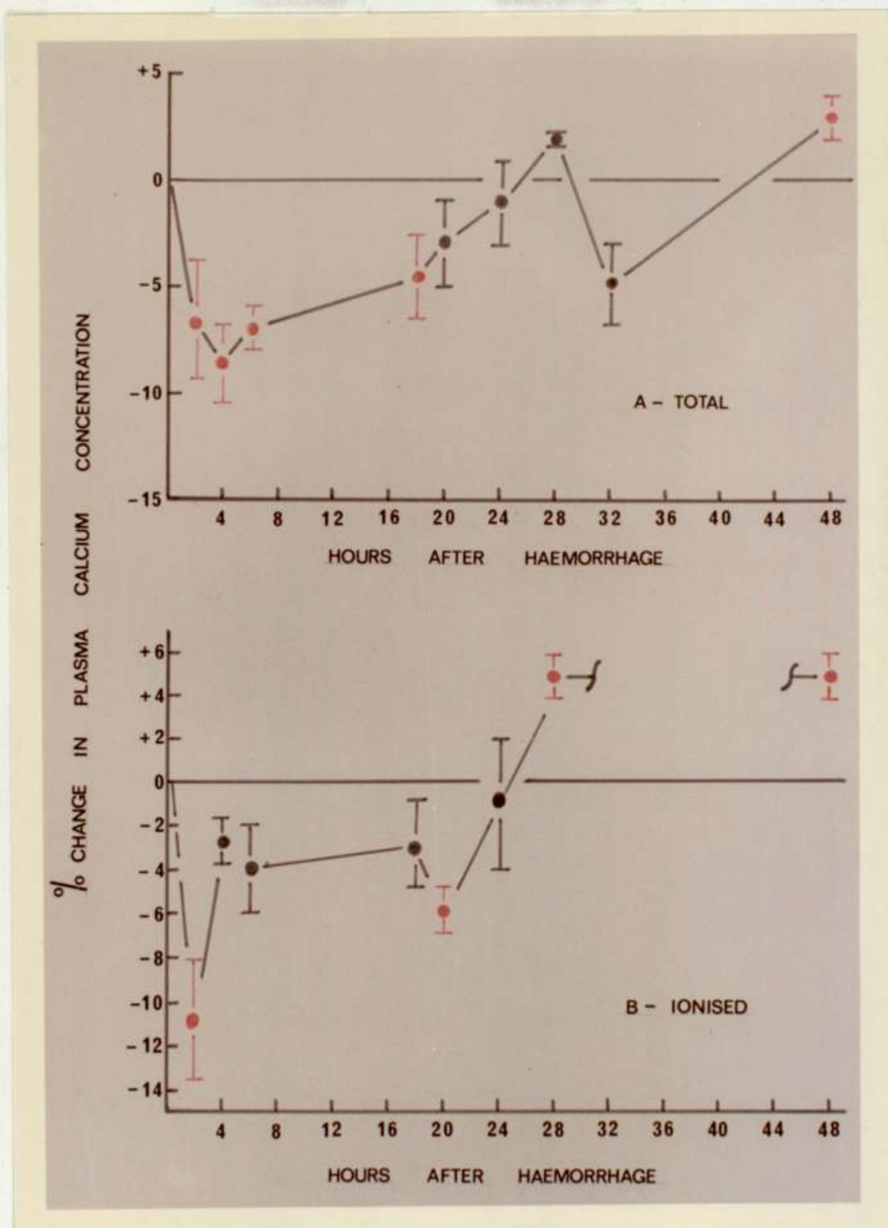


Figure 39: Plasma total (A) and ionised (B) calcium over the 48h period following haemorrhage in the rat. Blood (2.0 - 2.5ml/100g body weight) was removed by cardiac puncture at 0h. Changes in plasma calcium concentration are expressed as % of the control values. Each point is mean ± s.e.m. derived from the results from (A) 4-14 bled animals and 5-28 sham-bled controls and (B) 4-14 bled animals and 4-18 sham-bled controls. Points in red represent significant changes (P between < 0.05 and < 0.001 for both A and B).

Treatment		Haematocrit	Haemoglobin concentration (g/100ml)	Plasma protein concentration (g/100ml)
Sham bled	-4h	43.0 ± 1.5 (6)	13.5 ± 0.4 (6)	7.5 ± 0.3 (6)
Bled	-0h	42.5 ± 1.0 (17)	13.1 ± 0.3 (8)	7.7 ± 0.3 (7)
	-4h	32.0 ± 2.0 (8)	10.0 ± 0.3 (4)	6.2 ± 0.3 (4)

Data plotted as Figure 40, overleaf. Numbers of animals given in parentheses.

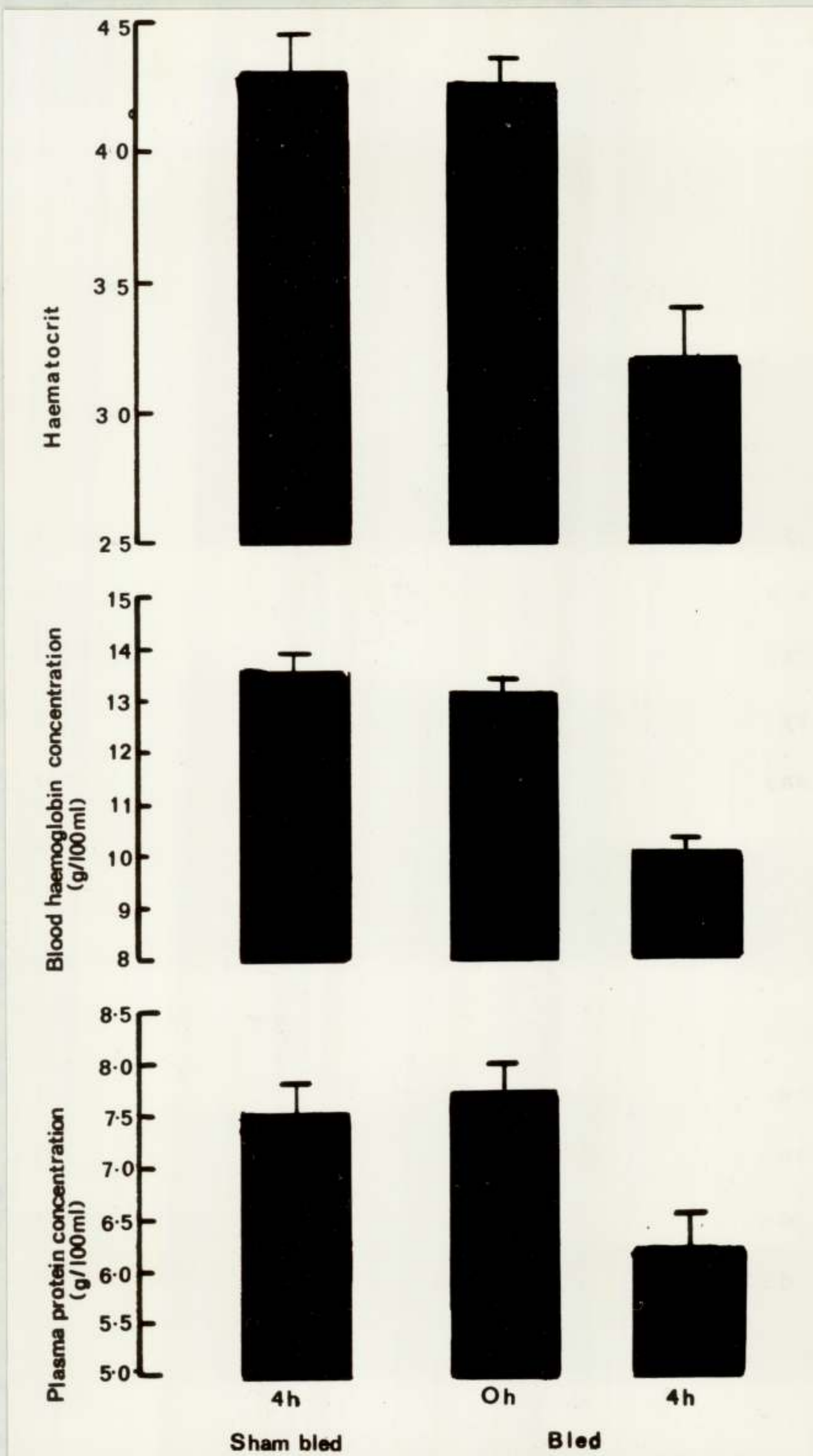


Figure 40: Haematocrit, haemoglobin concentration and plasma protein concentration 4h after haemorrhage in rats. Animals were bled (2.0-2.5ml/100g body weight) or sham bled at 0h. Significant decreases were observed in haematocrit ($P < 0.001$), blood haemoglobin concentration ($P < 0.001$) and plasma protein concentration ($P < 0.02$). Columns and vertical bars represent means + s.e.m. from 4-17 animals in each case.

Results.

One day after bleeding haematocrit had decreased by 30% (Figure 36). Since the volume of blood removed constituted some 30-35% of the total blood volume (Perris, personal communication) it is clear that blood volume had been restored within 24h. Haematocrit remained significantly depressed for 7 days after haemorrhage, indicating that there was probably a continuing erythropoietic demand over this period. Haematocrit was essentially re-established by the 8th day, which is 2 days longer than necessary in SPF rats (Perris et al., 1971).

The erythropoietic stimulus provided by the loss of circulating red cells was followed by an increase (presumably compensatory) in the mitotic index of the bone marrow (Figure 37). This must be considered to represent a true elevation of mitotic activity because it parallels the pattern seem by Perris and associates using Colcemid, which demonstrates the rate of entry of cells into division and their accumulation at metaphase. The increase in bone marrow mitotic index was maximal on days 3 and 4 and had disappeared by day 9 (Figure 37). Plasma total calcium concentration was also significantly elevated on days 2 - 7, but not on days 8 or 9 (Figure 38). Where measurements of plasma ionised calcium were made the pattern was similar (data not shown in Figure 38 but see 48h value in Figure 39).

Measurements of the plasma total and ionised calcium concentrations over a 2 day period after haemorrhage showed that there was a protracted hypocalcaemia which was not significantly reversed until 48h (Figure 39), at which time a hypercalcaemia was evident. The lowering of plasma calcium concentration could have been due to dilution of the blood remaining after haemorrhage by tissue fluids containing low concentrations of calcium, for the reductions in haematocrit, blood haemoglobin and plasma protein concentration at 4h (Figure 40) indicated that blood

Treatment	Plasma Concentration (mg/100ml)					
	Total Calcium			Inorganic Phosphate		
	0h	4h	P	0h	4h	P
Sham-bled	-	10.55±0.1 (4)	-	-	7.9±0.4 (4)	-
Bled	10.5±0.0 (5)	9.9 ±0.2 (5)	<0.02	8.3±0.4 (5)	7.7±0.2 (5)	>0.1
Bled-reinfused with whole blood	10.6±0.1 (6)	10.5 ±0.1 (6)	>0.5	7.8±0.2 (6)	8.0±0.3 (6)	>0.5
Bled-reinfused with plasma	10.6±0.1 (6)	10.1 ±0.1 (6)	<0.02	7.6±0.3 (6)	7.1±0.3 (6)	>0.5

Data plotted as Figure 41A/B overleaf. Numbers of animals indicated in parentheses.

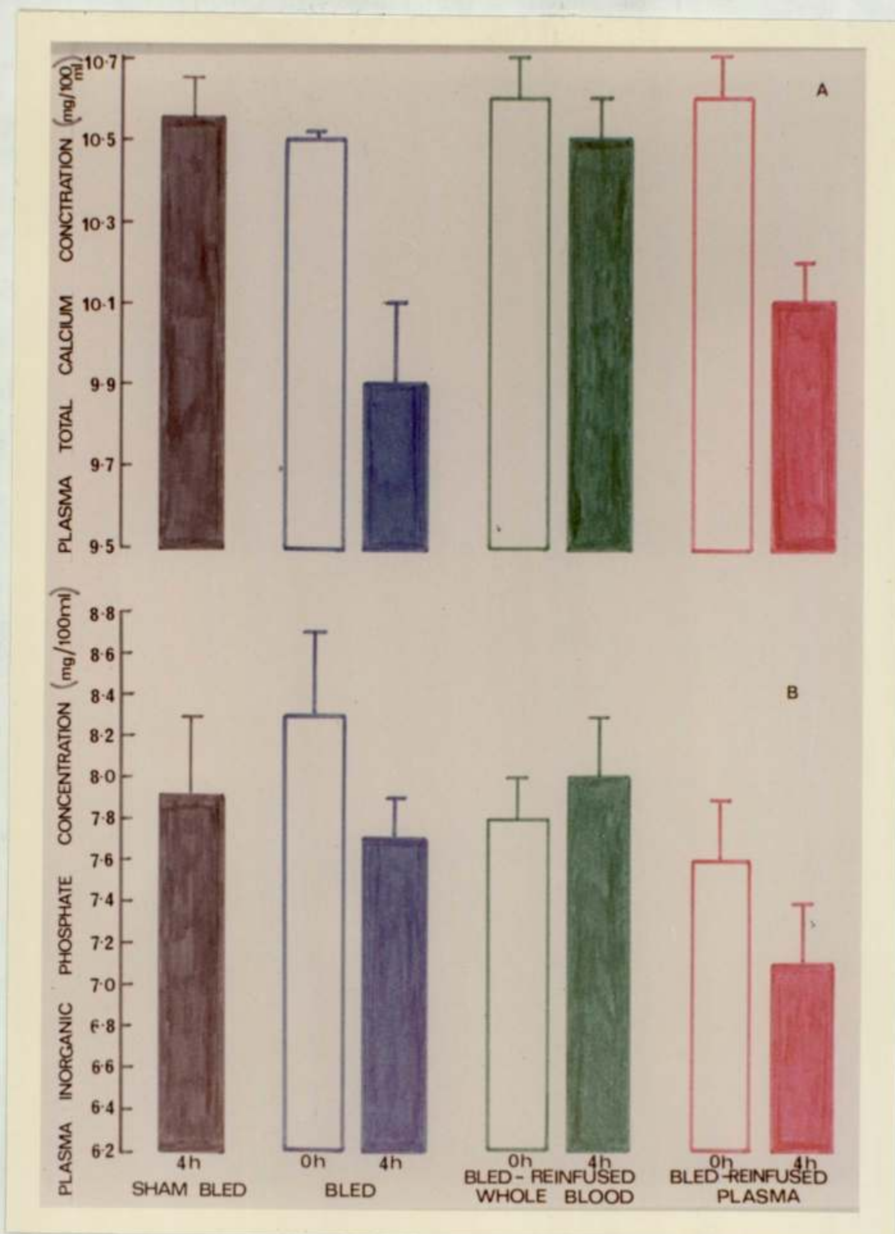


Figure 41: Effect of bleeding and bleeding with immediate reinfusion of homologous plasma or whole blood upon plasma total calcium and inorganic phosphate concentrations at 4h in rats. Blood (2.0-2.5ml/100g body weight) was removed by cardiac puncture at 0h. Control animals were sham-bled. Plasma total calcium concentration was significantly reduced in bled rats ($P < 0.01$) and in bled rats reinfused with homologous plasma ($P < 0.01$) but did not significantly change in bled rats reinfused with homologous whole blood ($P > 0.5$). Plasma inorganic phosphate concentration did not change significantly in any group ($P > 0.1$). Columns and bars represent means ± s.e.m. from 4-6 rats in each case.

Treatment	Plasma Calcium Concentration (mg/100ml)					
	Total			Ionised		
	0h	4h	P	0h	4h	P
Normal + Bleeding	10.4±0.1(5)	9.7±0.2(5)	<0.01	5.10±0.05(5)	4.9±0.1(5)	>0.1
Normal + Sham Bleeding	-	10.5±0.2(3)	-	-	5.10±0.1(3)	-
PTX + Bleeding	6.5±0.2(5)	5.95±0.1(5)	<0.05	2.60±0.1(5)	2.40±0.1(5)	>0.1
PTX + Sham-Bleeding	-	6.45±0.1(4)	-	-	-	-
Sham PTX + Bleeding	10.3±0.1(4)	9.8±0.2(4)	<0.05	5.00±0.05(4)	4.85±0.05(4)	>0.05
Sham PTX + Sham-Bleeding	-	10.3±0.1(3)	-	-	-	-

Data plotted as Figure 42A/B, overleaf. Numbers of animals given in parenthesis.

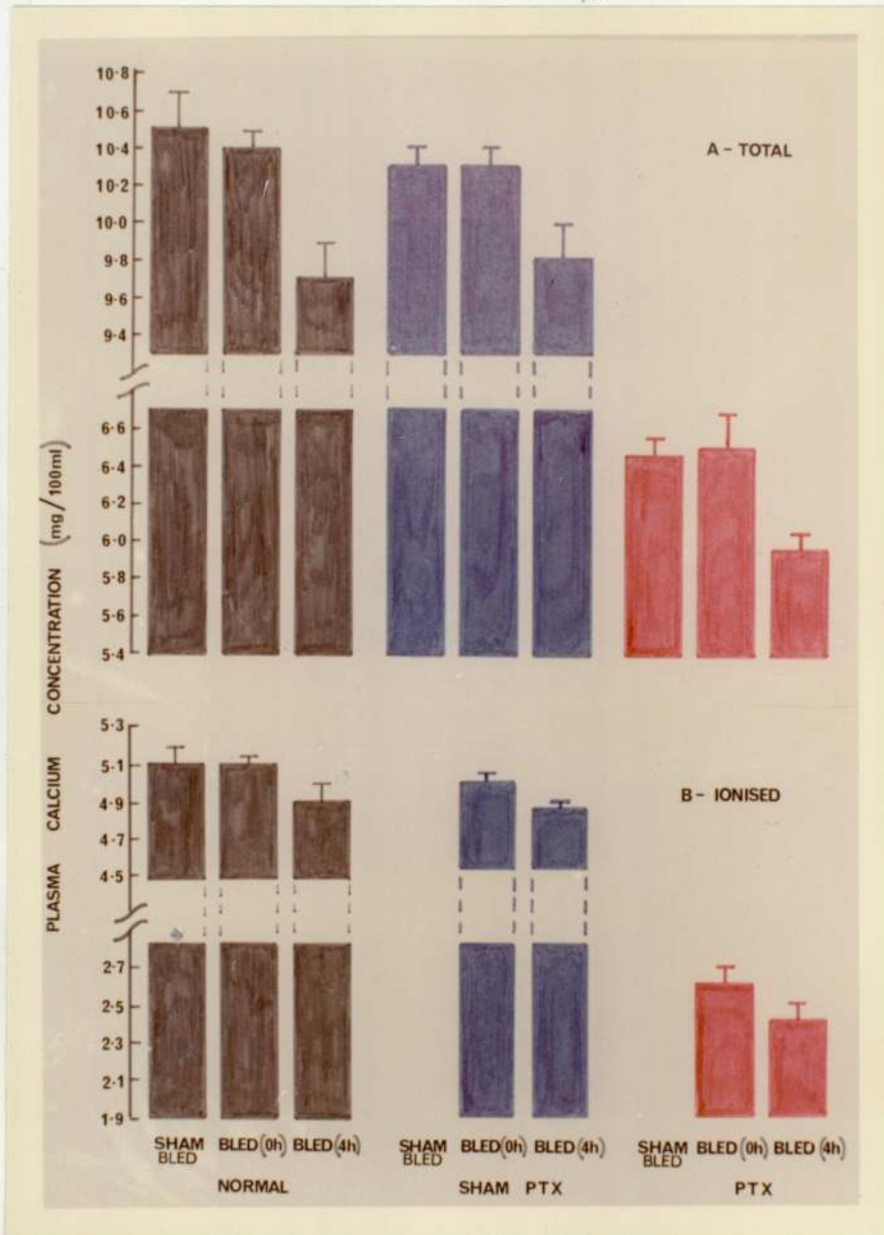


Figure 42: Effect of bleeding upon plasma calcium concentrations in intact, PTX and sham-PTX rats at 4h. The concentrations of total and ionised calcium in the plasma were measured 4h after removal of blood (2.0-2.5ml/100g body weight). Control animals were sham-bled. Plasma total calcium was significantly reduced in intact ($P < 0.01$), sham PTX ($P < 0.05$) and PTX ($P < 0.05$) rats. Ionised calcium in the plasma did not change significantly in any group ($P > 0.1$). Columns + vertical bars represent means + s.e.m. from 3-5 animals in each case.

Table 30 overleaf.

Treatment		Plasma calcium concentration (mg/100ml)	
		Total	Ionised
Bled-reinfused control blood	- 0h	10.3 ± 0.1 (9)	4.90 ± 0.10 (3)
	- 4h	10.2 ± 0.2 (8)	4.80 ± 0.10 (3)
Bled-reinfused CO-treated blood	- 0h	10.25 ± 0.1(16)	4.85 ± 0.05 (11)
	- 4h	10.0 ± 0.2(14)	4.65 ± 0.10 (7)

Table 30: Plasma calcium concentration 4h after bleeding and reinfusion with control or carbon monoxide (CO) treated blood. Blood (2.0-2.5ml/100g body weight) was removed by cardiac puncture at 0h and immediately replaced by reinfusion of control blood or blood containing CO-saturated red cells. See Methods, Supplement 2 for preparation of reinfusates and Methods (main text, this chapter) for description of bleeding and reinfusion. Neither plasma total or ionised calcium concentrations were significantly affected by these procedures ($P > 0.1$ in all cases). Columns and vertical bars represent means + s.e.m. from 3-16 animals in each case.

volume had been essentially restored at this time. This agrees with the observations of Pareira, Serkes and Lang (1960) in the rat. However, a significant hypocalcaemia was also observed in animals which had been reinfused immediately after bleeding with homologous donor plasma, but not in those which had received whole blood from the same source (Figure 41), suggesting that the loss of red blood cells was in some way responsible for the greater part of the observed decrease in plasma total calcium concentration at 4h. Plasma inorganic phosphate concentration did not change in any of these situations (Figure 41). The hypocalcaemia could not have been produced by a sudden cessation of PTH secretion because it was also evident in bled PTX rats as well as their sham-operated bled controls (Figure 42). Reinfusion of blood containing CO-saturated red cells, to produce anoxia without affecting blood cellularity or volume, caused only a small decrease in plasma total calcium concentration which was not significant (Table 30). Thus it was doubtful that the fall in plasma calcium concentration immediately after haemorrhage was a result of hypoxia produced by loss of circulating red cells and hence total oxygen carrying capacity.

Although high circulating levels of EPO are evident during the first 48h following haemorrhage (Jacobson & Goldwasser, 1958; Fried _et al._, 1970) it should be recalled that the hypercalcaemia which this hormone provokes is slow to develop after its exogenous administration and that until it does so no erythropoietin-dependent increases in bone marrow mitotic activity are evident (Chapter 2). It seems reasonable to assume that the endogenous hormone cannot stimulate mitotic activity in the bone marrow until the hypercalcaemic phase is well established at 48h and beyond. After haemorrhage, however, there are two periods where heightened mitotic activity exists (at 4 and 18h) long before the EPO-induced hypercalcaemic phase is prominent. Furthermore, these two

Hours After Haemorrhage or Sham-Bleeding	Bone Marrow Mitotic Index				
	Sham-Bled	Bled	Δ	P	% Change
2	0.85±0.1 (4)	1.05±0.2 (4)	(+)0.2	>0.5	(+)23.5±23.5
4	1.2 ±0.1(16)	1.8 ±0.1(21)	(+)0.6	<0.001	(+)50.0± 8.3
6	1.2 ±0.1 (4)	1.25±0.1 (5)	(+)0.05	>0.5	(+) 4.2± 8.4
18	1.0 ±0.1 (5)	1.55±0.1 (5)	(+)0.55	<0.001	(+)55.0±10.0
20	1.55±0.2 (4)	1.6 ±0.3 (5)	(+)0.05	>0.5	(+) 3.2±19.2
24	1.3 ±0.2 (6)	1.7 ±0.2 (4)	(+)0.4	>0.1	(+)30.8±15.4
28	1.1 ±0.1 (4)	1.4 ±0.1 (5)	(+)0.3	<0.05	(+)27.3± 9.1
32	1.8 ±0.2 (5)	1.55±0.1 (6)	(-)0.25	>0.1	(-)13.9± 5.6
48	1.3 ±0.1 (5)	1.9 ±0.1 (6)	(+)0.6	<0.01	(+)46.2± 7.7

Data plotted as Figure 43A/B, overleaf. Numbers of animals given in parentheses.

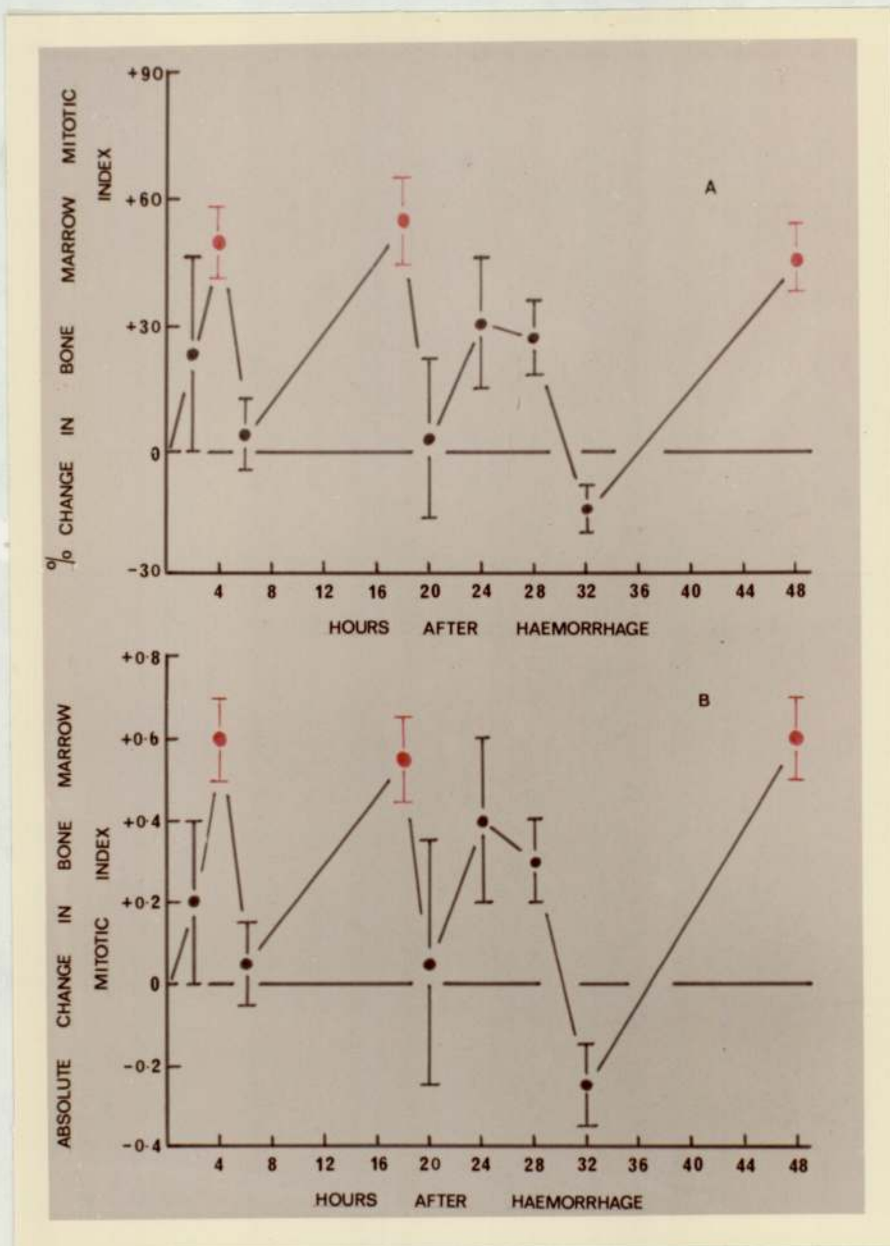


Figure 43: Bone marrow mitotic index 0-48h after haemorrhage in rats. Animals were bled (2.0-2.5ml/100g body weight) or sham-bled at 0h. Changes in marrow mitotic index are represented as % of control values (A) or as absolute values (B). Points in red indicate significant changes ($P < 0.01$ or $P < 0.001$). Points and vertical bars represent means ± s.e.m. derived from 4-21 bled rats and 4-16 sham-bled controls.

Treatment	Plasma Total Calcium Concentration (mg/100ml)	Bone Marrow Mitotic Index
Saline	10.1 ± 0.1 (10)	1.45 ± 0.3 (10)
Aldosterone	10.0 ± 0.1 (5)	1.20 ± 0.3 (5)
Angiotensin II	9.8 ± 0.2 (5)	1.10 ± 0.2 (5)
ADH	10.25 ± 0.2 (5)	2.2 ± 0.2 (5)

Data plotted as Figure 44A/B, overleaf. Numbers of animals given in parentheses.

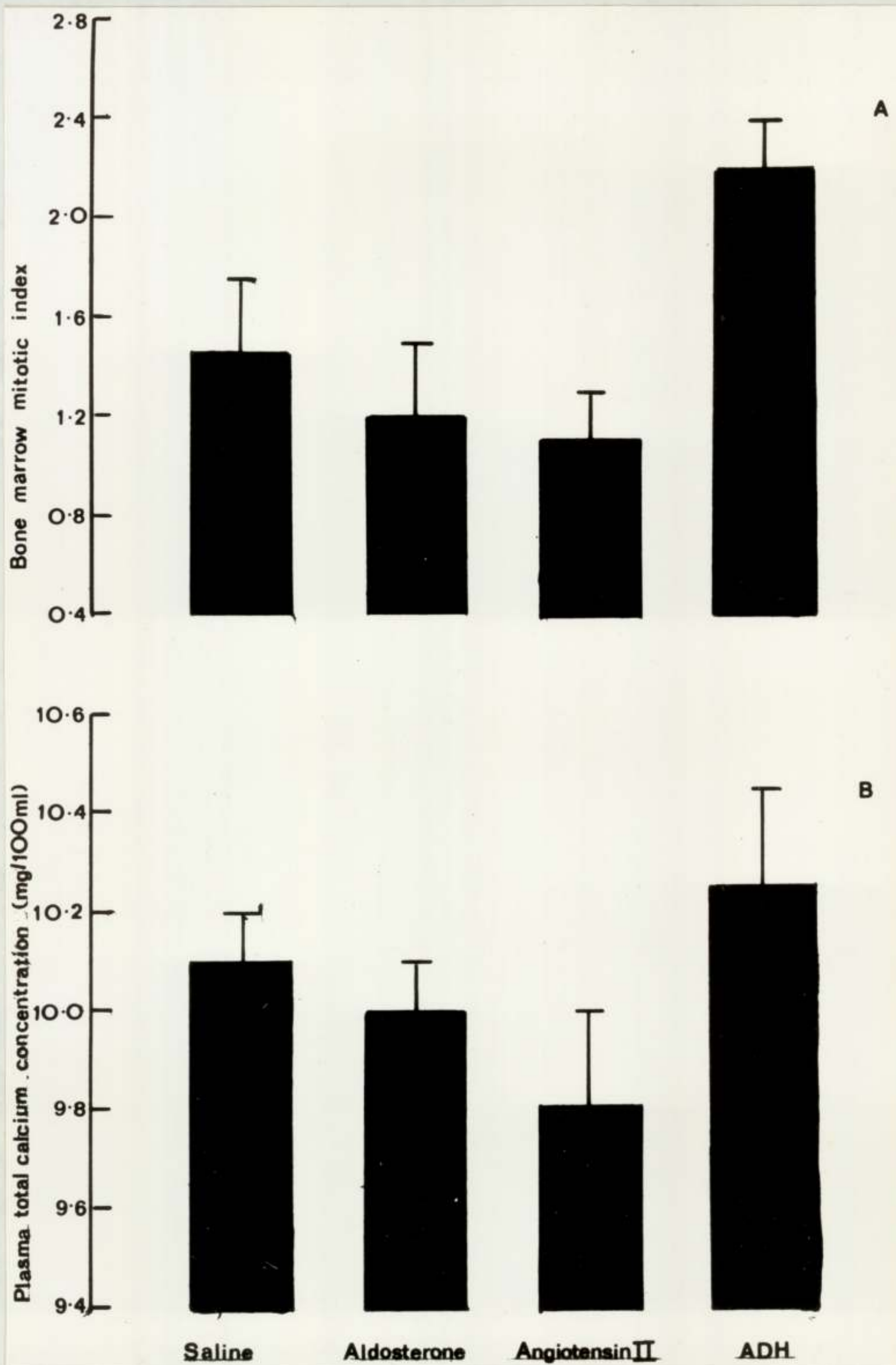


Figure 44: Effects of aldosterone, angiotensin II and anti-diuretic hormone (ADH) upon bone marrow mitotic index and plasma total calcium concentration at 4h in rats. The hormones were dissolved in 0.9% saline and administered by various routes (see text for rationale) at 0h. Doses were equivalent to those released endogenously after haemorrhage (see Methods and Discussion). ADH significantly elevated (A) bone marrow mitotic index ($P < 0.01$), whereas aldosterone and angiotensin did not ($P > 0.1$). Plasma total calcium concentration (B) did not change significantly in any group ($P > 0.1$). Columns + vertical bars represent means ± s.e.m. from 5 - 10 rats in each case.

Tables 31 and 32 overleaf.

Treatment	Bone Marrow Mitotic Index at 4h
ADX + Bleeding	2.5 ± 0.1 (7)
ADX + Sham-Bleeding	1.7 ± 0.1 (5)
P	$<$0.001

Table 31: Effect of haemorrhage upon bone marrow mitotic index in adrenalectomised (ADX) rats at 4h. Blood (2.0-2.5ml/ 100g body weight) was removed at 0h. Control animals were sham-bled. Marrow mitotic index was 47% higher in bled than in sham-bled animals. Numbers of animals given in parentheses.

Treatment	% Nucleated Bone Marrow Cells in Metaphase at 4h following Colcemid
Saline	9.2 ± 0.4 (5)
ADH	12.8 ± 0.5 (5)
P	$<$0.001

Table 32: Effect of anti-diuretic hormone (ADH) upon bone marrow mitotic activity at 4h in rats. ADH (6mU/100g body weight) was injected intracardially (i.c.) in 0.1ml 0.9% saline at 0h. Controls received 0.1ml 0.9% saline i.c. The percentage of nucleated bone marrow cells trapped at metaphase by Colcemid (dose and protocol in Methodology, Section 6.4) was 40% higher in ADH-injected animals than in controls. Numbers of animals given in parentheses.

surges in mitosis occur in the face of hypocalcaemia, which might be expected to lessen cell division. These early increases in mitosis must, therefore, be ascribed to factors other than EPO and elevation of extracellular calcium concentration.

The cause of the mitotic peak at 18h was not investigated, but bone marrow mitotic activity was measured 4h after the administration of aldosterone, angiotensin II and ADH in doses equivalent to their endogenous post-haemorrhagic levels. Injection of aldosterone and angiotensin did not significantly alter bone marrow mitotic index, whereas ADH produced a highly significant ($P < 0.01$) increment (Figure 44A). This action was independent of changes in plasma calcium concentration (Figure 44B) and the presence of the parathyroid gland (discussed later).

Although injection of aldosterone and angiotensin did not appear to stimulate bone marrow mitosis (Figure 44A) it was clear that a number of hormones of adrenal origin which were known to be secreted in supranormal quantities after haemorrhage (see Introduction) could not be eliminated as possible mitogens. For example, both cortisol and adrenaline stimulate cellular proliferation in cultures of thymic lymphocytes (Whitfield et al., 1970c; MacManus et al., 1971a). Bone marrow mitotic index was therefore measured 4h after bleeding in ADX rats (Table 31) and was again found to be significantly increased ($P < 0.001$). Thus the adrenal hormones did not appear to significantly contribute to the bone marrow response to haemorrhage at 4h.

To demonstrate that ADH produced a true increase in the rate of entry of bone marrow cells into division, Colcemid was administered to rats immediately after the hormone (Table 32). The percentage of nucleated bone marrow cells which had reached metaphase in 4h was significantly higher ($P < 0.001$) in animals which had received ADH than in saline-treated controls. It was possible that the posterior

Treatment	Bone Marrow Mitosis at 4h	
	% Nucleated Cells in Metaphase After Colcemid	Mitotic Index
Saline + Mannitol (1.5pg/100g)	10.5 ± 1.2 (5)	0.95 ± 0.1 (5)
DDAVP (75pg/100g) + Mannitol (1.5pg/100g)	15.1 ± 0.9 (4)	1.6 ± 0.2 (5)
DDAVP (100pg/100g) + Mannitol (2pg/100g)	15.6 ± 1.0 (5)	-

Data plotted as Figure 45A/B, overleaf. Numbers of animals indicated in parentheses. DDAVP = (1-deamino-8-D-arginine)-vasopressin.

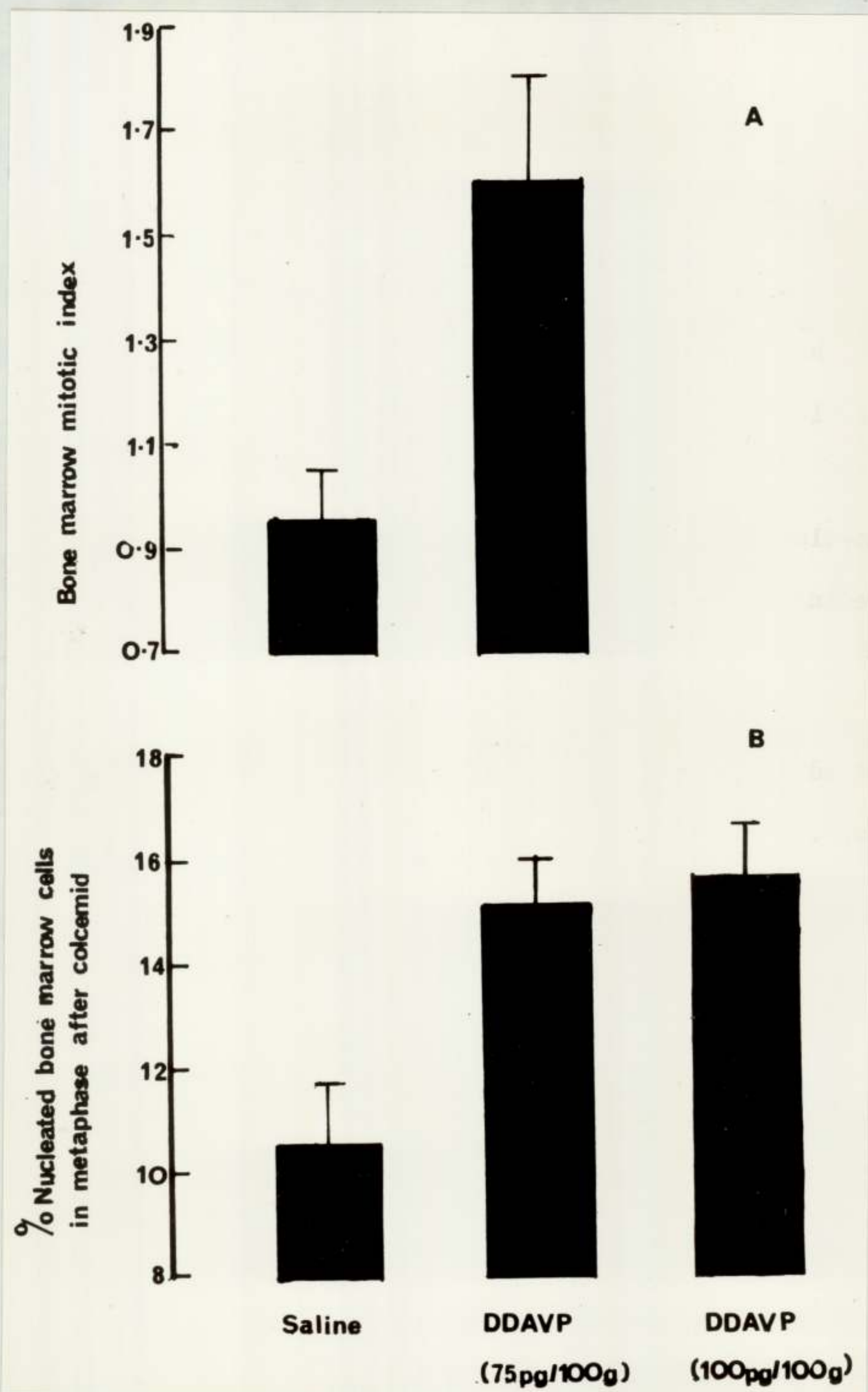


Figure 45: Effect of (1-deamino-8-D-arginine)-vasopressin (DDAVP) upon bone marrow mitotic activity at 4h in rats. DDAVP was dissolved in 0.1ml 0.9% saline and injected i.c. at 0h. Controls received saline plus 1.5pg mannitol/100g body weight, since 75pg of the DDAVP preparation contained this quantity of mannitol. The 75pg/100g dose of DDAVP (which had anti-diuretic activity equivalent to 6mU/100g body weight of ADH) significantly increased (A) bone marrow mitotic index ($P < 0.001$) and (B) metaphase accumulation after Colcemid ($P < 0.01$). The 100pg/100g dose also significantly increased the % of nucleated bone marrow cells trapped in metaphase after Colcemid ($P < 0.01$). Columns and vertical bars represent means + s.e.m. from 4-5 animals in each case. See Discussion for derivation of dosage and route of administration of DDAVP.

Treatment	Bone Marrow Mitosis at 4h	
	% Nucleated Cells in Metaphase After Colcemid	Mitotic Index
Control + Sham-Bleeding	9.9 ± 0.8 (4)	1.20 ± 0.0 (3)
Control + Bleeding	12.5 ± 0.5 (4)	1.45 ± 0.1 (5)
HX + Sham-Bleeding	7.9 ± 1.1 (4)	0.85 ± 0.1 (5)
HX + Bleeding	3.5 ± 1.6 (6)	0.5 ± 0.2 (7)

Data plotted as Figure 46A/B, overleaf. Numbers of animals given in parentheses. HX = hypophysectomised.

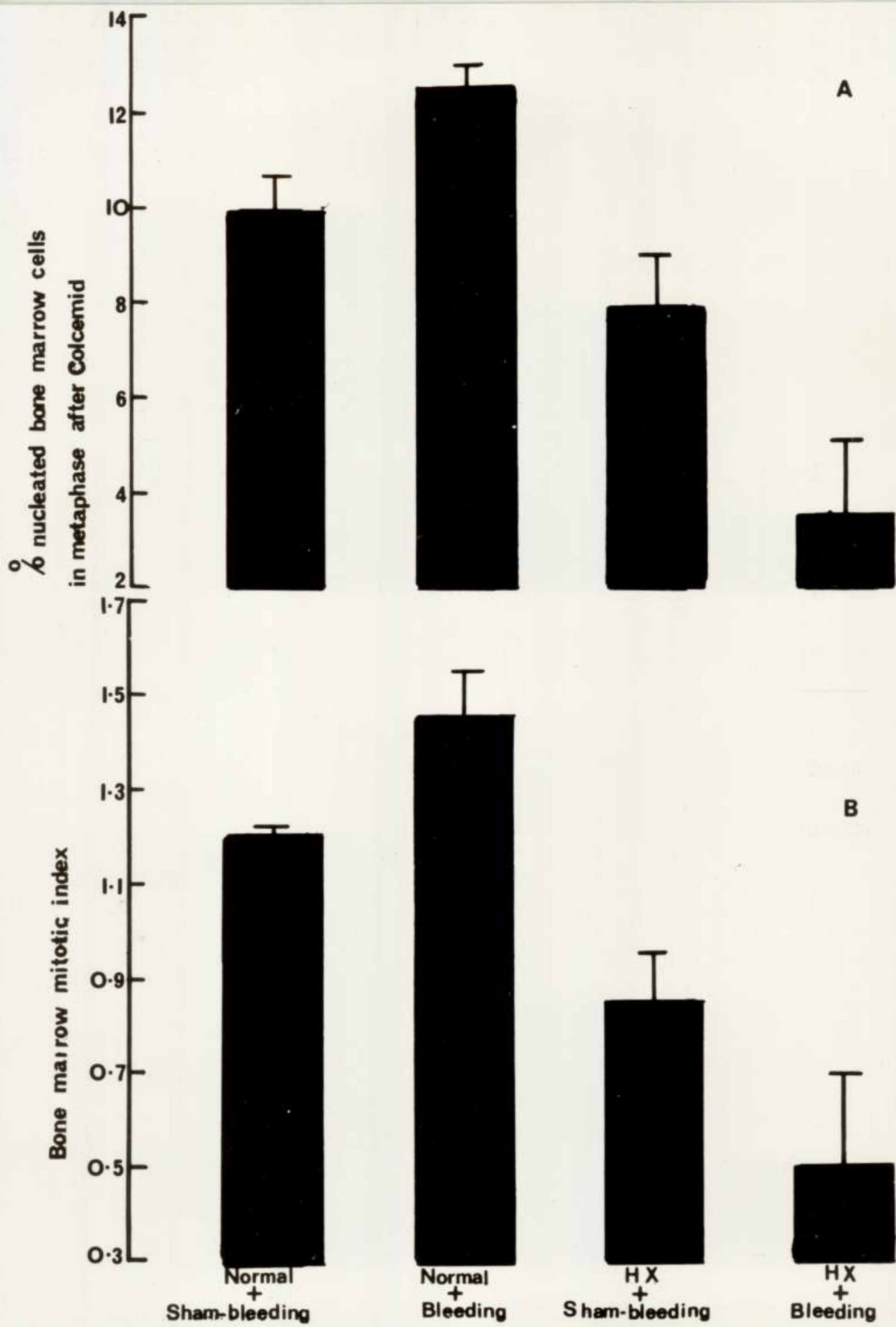


Figure 46: Effect of bleeding upon bone marrow mitotic activity at 4h in intact and hypophysectomised (HX) rats. Blood (2.0-2.5ml/100g body weight) was removed by cardiac puncture from normal and HX rats at 0h. Control animals were sham-bled. Haemorrhage significantly increased the % of nucleated bone marrow cells trapped in metaphase after Colcemid (A) and the marrow mitotic index (B) in intact animals ($P < 0.05$ in both cases). However, both these parameters decreased in bled HX rats, although only the decrease in Colcemid-metaphase accumulation was significant ($P < 0.001$). Columns and vertical bars represent means + s.e.m. from 3-7 animals in each case.

Treatment	Plasma Concentration (mg/100ml)	
	Total Calcium	Inorganic Phosphate
Control + Sham-Bleeding	10.1 ± 0.1 (3)	8.8 ± 0.1 (3)
Control + Bleeding	9.6 ± 0.1 (4)	8.3 ± 0.3 (4)
HX + Sham-Bleeding	9.6 ± 0.2 (5)	6.3 ± 0.2 (4)
HX + Bleeding	9.1 ± 0.1 (7)	6.9 ± 0.4 (7)

Data plotted as Figure 47, overleaf. Numbers of animals given in parentheses. HX= hypophysectomised.

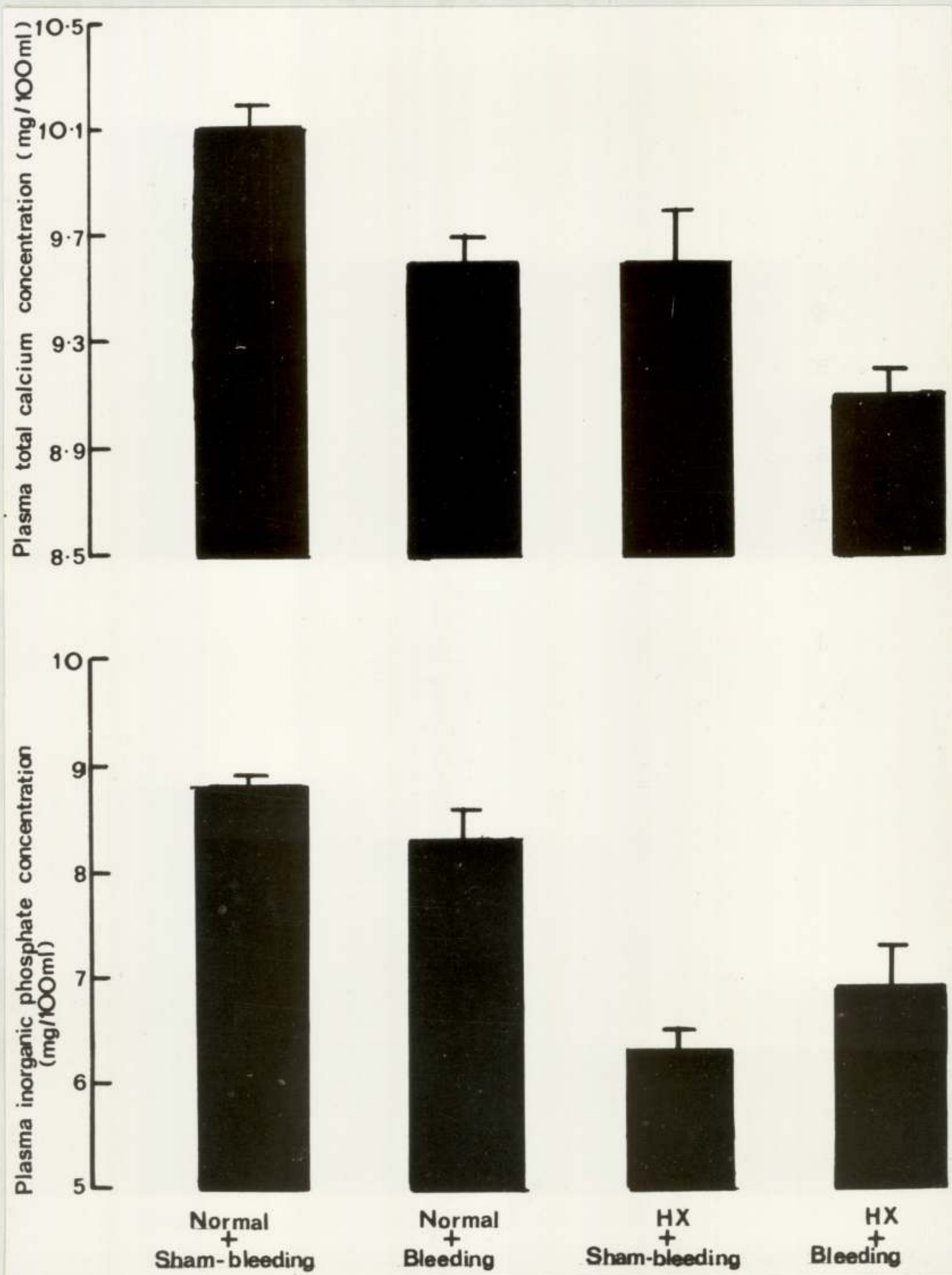


Figure 47: Effect of haemorrhage upon the plasma concentrations of total calcium and inorganic phosphate at 4h in intact and hypophysectomised (HX) rats. Animals were sham-bled or bled (2.0-2.5ml/100g) by cardiac puncture at 0h. Plasma total calcium concentration (A) was significantly reduced in both normal and HX rats after haemorrhage ($P < 0.05$ in both cases). The concentrations of inorganic phosphate in the plasma (B) were not, however, changed ($P > 0.1$ in both cases). Columns and vertical bars represent means + s.e.m. from 3-7 animals in each case.

Treatment	Bone Marrow Mitotic Index at 4h	P
Normal + Sham-Bleeding	1.0 ± 0.2 (3)	< 0.01
Normal + Bleeding	1.5 ± 0.1 (5)	
Sham PTX + Sham-Bleeding	0.9 ± 0.1 (3)	< 0.001
Sham PTX + Bleeding	1.5 ± 0.2 (5)	
PTX + Sham-Bleeding	0.4 ± 0.1 (4)	< 0.001
PTX + Bleeding	1.0 ± 0.1 (5)	

Data plotted as Figure 48, overleaf. Numbers of animals given in parentheses.

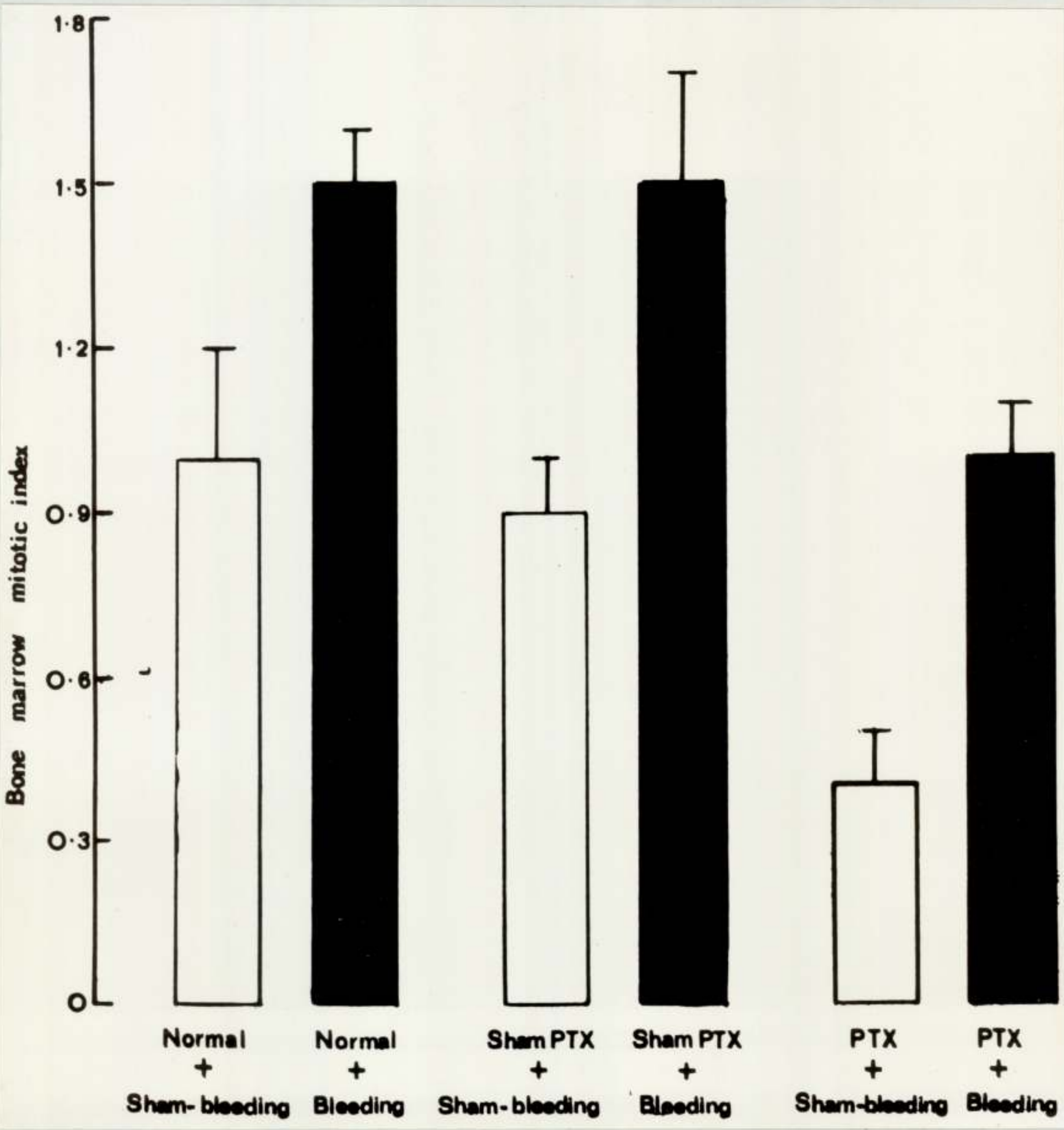


Figure 48: Effect of haemorrhage upon bone marrow mitotic index at 4h in intact, sham-PTX and PTX rats. Animals were bled (2.0-2.5ml/100g) by cardiac puncture or sham-bled at 0h. After 4h, bone marrow mitotic index was significantly elevated in normal ($P < 0.01$), sham-PTX ($P < 0.001$) and PTX ($P < 0.001$) rats. Columns and vertical bars represent means + s.e.m. from 3-5 rats in each case.

Table 33 overleaf.

Treatment	% nucleated bone marrow cells in metaphase (Colcemid present)	
	4h	6h
Untreated	2.8 ± 0.2 (5)	4.8 (2)
ADH (0.3mU/ml)	4.1 ± 0.3 (5)	6.8 (2)
ADH (0.6mU/ml)	4.0 ± 0.2 (5)	7.0 (2)
ADH (1.2mU/ml)	3.0 (2)	-
EPO (0.25u/ml)	3.3 ± 0.2 (4)	5.2 (2)
EPO (o.5u/ml)	3.4 ± 0.3 (4)	5.0 (2)
EPO (1.0u/ml)	2.4 (2)	-
Aldosterone (2.5ng/ml)	2.7 (2)	-
Aldosterone (5ng/ml)	2.8 ± 0.1 (4)	4.6 (2)
Angiotensin II (1.5ng/ml)	2.9 (2)	-
Angiotensin II (3ng/ml)	2.6 ± 0.3 (4)	4.9 (2)

Table 33: Effect of hormones upon bone marrow mitotic activity *in vitro*. See General Methodology, Sections 7.3 and 7.4 for description of culture technique. Anti-diuretic hormone (ADH - 0.3mU/ml and 0.6mU/ml) significantly ($P < 0.05$ in both cases) increased the % of nucleated cells reaching metaphase after 4h in the presence of 0.062mM Colcemid. Erythropoietin (EPO), aldosterone and angiotensin II amide did not significantly affect bone marrow cellular proliferation ($P > 0.1$, $P > 0.5$ and $P > 0.5$, respectively). Numbers of duplicate cultures given in parentheses.

pituitary extract ("Pitressin") contained a contaminating mitogenic agent which was responsible for the observed elevation of bone marrow mitotic activity. Thus an extremely pure preparation of the ADH analogue DDAVP was injected into normal rats. This substance possesses potent anti-diuretic, but very little vasopressor, activity (Zaoral et al., 1967; Vavra, et al., 1968) and was injected in a dose range with anti-diuretic activity equivalent to that of the ADH previously administered (Vavra et al., 1968). DDAVP produced large increments in both bone marrow mitotic index and in the rate of accumulation of bone marrow cells in metaphase after Colcemid (Figure 45).

Since ADH is secreted from the posterior pituitary gland it was reasoned that HX rats might not exhibit increased bone marrow mitotic activity 4h after haemorrhage. This hypothesis was substantiated by the data shown in Figure 46. Indeed, cellular proliferation in the bone marrow actually decreased over the post-haemorrhagic period, in parallel with the decrease in plasma calcium which was simultaneously observed (Figure 47). Note that plasma inorganic phosphate concentration was again not significantly affected by haemorrhage in intact animals, nor in the HX rats (Figure 47).

The mitogenic activity of ADH did not depend upon the presence of the parathyroid gland; bleeding produced increases in bone marrow mitotic index at 4h in PTX, as well as intact and sham PTX, animals (Figure 48).

The demonstration that ADH, in contrast to aldosterone, angiotensin and EPO, could increase mitotic activity in bone marrow cells maintained in vitro for 4-6h (Table 33) strongly suggests that the hormone exerts its mitogenic action directly upon bone marrow elements.

Discussion.

The results of these studies, while confirming and elaborating the observations of Perris *et al.*, (1971), are consistent with the hypothesis that the sustained hypercalcaemia evident from 2-7 days after haemorrhage is at least partly dependent upon increased circulating levels of EPO. Furthermore it is clear that the peak of bone marrow mitotic activity which occurs at 4h, during a period of depressed plasma calcium concentration, is produced by a rapid surge in the circulating levels of ADH immediately after blood loss.

Blood volume is re-established within 4h of a severe haemorrhage in rats (Pareira *et al.*, 1960; Figure 40) and thus, if it is assumed that further haemodilution does not occur, it is clear that the subnormal haematocrit observed until the 7th day following bleeding (Figure 36) indicates the existence of a persistent erythropoietic demand over this period. Both bone marrow mitotic index and plasma calcium concentration were significantly elevated from day 2 to day 7 (Figures 37 and 38). This parallel, confirming that observed by Perris *et al.* (1971), suggests that the factors could be causally related, for there is abundant evidence which relates elevations in plasma calcium concentration to increases in bone marrow cellular proliferation (see Perris, 1971; Whitfield *et al.*, 1973c; General Introduction, Sections 5.2 and 5.6; Chapters 1 and 2).

Circulating EPO levels in rats have been demonstrated to rise rapidly after severe haemorrhage (Jacobson & Goldwasser, 1958; Fried *et al.*, 1970). The latter authors demonstrated that plasma EPO titres were still significantly supranormal 3 days after an exsanguination equivalent to that performed in the present studies. At that time the erythropoietic activity of the plasma of the bled rats was

some 6 times greater than that of normal animals, and approximately 30% of the peak value which occurred at 24h. Measurements of plasma EPO levels were not, however, made on subsequent days. Since injection of exogenous EPO increases plasma calcium concentrations (Chapter 2) it is possible that circulating endogenous EPO produced the sustained hypercalcaemia observed in the present study (Figure 38). Stimulation of the release of PTH by the initial hypocalcaemic phase, as suggested by Perris et al. (1971), could contribute to this action for EPO has been postulated to act synergistically with PTH at the latter's target sites of action (Chapter 2). However, since circulating levels of the indirectly hypercalcaemic hormone EPO are high over the first 48h after haemorrhage (Fried et al., 1970) while plasma calcium concentrations are generally subnormal (Figure 39), it is clear that a strong hypocalcaemic stimulus must follow blood loss.

Bone marrow mitotic activity was still significantly elevated on the 8th day after haemorrhage (Figure 37), when plasma calcium concentrations (Figure 38) and haematocrit had returned to normal and erythropoietic demand was presumably small (Figure 36). At this time however a need for increased white blood cell production may still exist. During acute erythropoietic challenge proliferation in the non-erythroid bone marrow compartments may be reduced (Hellman & Grate, 1967), probably because of stem cell competition. It was reasoned that the resultant deficiencies in white blood cells, blood platelets or lymphocyte precursors might continue to stimulate bone marrow mitosis after restoration of the haematocrit. Preliminary experiments have indicated that there is a deficiency in circulating white blood cells on the 7th and 8th days after haemorrhage. It should be noted that the granuloid series appears not to be influenced by the calcium homeostatic system, for this compartment, unlike the erythroid and lymphoid compartments, does not exhibit hypoplasia after removal of the parathyroids (Rixon & Whitfield, 1972a). The possibility that the disturbances in circul-

ating white cell numbers were produced by infection rather than the mechanism suggested above cannot be excluded from consideration and thus the preliminary indication which has been reported should be treated cautiously. This observation must be repeated in SPF rats in aseptic conditions to ensure that the granuloid demand after the erythroid demand has diminished is a real one.

The crucial observation (Perris et al., 1971) that the hypocalcaemic phase following haemorrhage was a consequence of the loss of red cells, rather than blood volume, has been confirmed (Figure 41), with the slight refinement that control animals were reinfused with donor, rather than their own, blood. Since bled animals drink very little over the first 4h after haemorrhage (personal observation) it is likely that plasma volume is restored from interstitial fluid, in which the concentration of calcium is some 10-15% less than that of plasma (Rasmussen, 1970), or intracellular fluid, which contains little free calcium (Borle, 1967, 1968). However, this dilution factor appears to contribute only partially to the decrease in plasma calcium concentration observed at 4h (compare Figures 39 and 41). Since removal of red cells causes a reduction in the tissue oxygen supply it has been postulated (Perris et al., 1971) that the active metabolic processes which maintain free intracellular calcium concentrations at low levels (Borle, 1967, 1968) might thus be inhibited and allow a surge of calcium into the tissues. Metabolic inhibitors certainly do have this effect in certain tissues (Schachter, Kinberg & Schenker, 1961; Passow, 1963; Wallach, Reizenstein, & Bellavia, 1966). The rapid fall in plasma ionised calcium observed over the first 2h following haemorrhage (Figure 39B), which accounts for almost all of the decrease in total plasma calcium at this time, would also tend to support this hypothesis. However, replacement of whole blood by homologous donor blood containing red cells in which

the oxygen-carrying capacity had been abolished by saturation with CO did not significantly depress plasma calcium concentrations (Table 30). Animals thus treated might be expected to experience hypoxia without concurrent changes in the viscosity and flow dynamics of the blood. Assuming that the experimental design did produce this situation, these data would militate against the concept that hypoxia had produced hypocalcaemia by the mechanism discussed above. It might, however, be argued that CO could have become displaced from erythrocyte haemoglobin and opposed the hypothetical scheme outlined above by some mechanism which is not immediately apparent. This seems unlikely. The possibility remains that changes in blood flow due to the loss of red cells might produce the hypocalcaemia by a mechanism that remains obscure. The calcium homeostatic hormones PTH and CT are clearly not essential for the development of the phenomenon for it is observed in PTX (Figure 42) and TPTX (Perris et al., 1971) animals. This conclusion is further substantiated by the absence of any change in plasma inorganic phosphate concentration over the first 4h after haemorrhage (Figure 41). It seems most likely that the hypocalcaemia results from physicochemical interactions which are specific to calcium and are triggered by the loss of red blood cells.

It should be noted that a significant depression of plasma ionised calcium concentration 4h after haemorrhage, which was reported by Perris et al., (1971), has not been observed in the current experiments, although the concentration of total calcium was significantly ($P < 0.001$) reduced (compare A and B, Figure 39). The relative increase in the proportion of plasma calcium present in the ionised form is understandable because plasma protein concentration is some 25% lower than normal at this time (Pareira et al., 1960; Figure 40). Furthermore, the amount of CO_2 dissolved in the plasma might well increase during

this period of red cell deficiency; if, despite buffering mechanisms, this factor lowered blood pH (Bowman, Rand & West, 1968) the ionised calcium fraction in plasma would increase (see General Methodology, Sections 5.2, 5.3 and 7.4 for discussion). Presumably differences in the strains of rats which were used could explain the discrepancies between the present observations and those of Perris and his colleagues. The decrease in ionised calcium reported by them was, however, only some 6% of the control value, compared with a 13% reduction in total calcium in the plasma (c.f. Figure 39).

During the hypocalcaemic phase, however it is caused, two peaks of bone marrow mitotic activity occurred at 4 and 18h (Figure 43) which could not be attributed to high circulating levels of endogenous EPO since this hormone does not increase marrow cellular proliferation in the absence of elevated mobile calcium. Of the hormonal factors which could be implicated ADH, aldosterone, adrenaline, cortisol and angiotensin deserve attention as their levels are very significantly elevated (albeit fairly briefly) after haemorrhage. The doses and routes of administration of these hormones were designed to replicate their patterns of release after haemorrhage, and were derived from the data of Ginsburg & Heller, 1953; Davis, 1961; Bojeson, 1964; Eilers & Peterson, 1964; Muller <u>et al.</u>, 1964; Scornik & Paladini, 1964; Lodge <u>et al.</u>, 1966; Share, 1968; Errington & Rocha e Silva, 1971; Johnson <u>et al.</u>, 1971; Cousineau <u>et al.</u>, 1973. Some of the quoted studies were performed in dogs, but wherever possible the relevance of the results to rats has been confirmed from the literature. Aldosterone and angiotensin II had no mitogenic activity in bone marrow either <u>in vivo</u> or <u>in vitro</u> at the dose levels tested (Figure 44 and Table 33). Aldosterone injection has been claimed to stimulate erythropoiesis (Cooper <u>et al.</u>, 1968), although other workers (Mann <u>et al.</u>, 1966;

Zivny et al., 1972) have been unable to substantiate such findings. The present studies suggest that aldosterone does not increase erythropoietic activity. Angiotensin II has been reported to stimulate erythropoiesis in the rabbit (Nakao et al., 1967) although it clearly did not stimulate bone marrow mitosis in the experiments reported here (Figure 44 and Table 33).

Since removal of the adrenal glands did not abolish the increase in mitotic index observed 4h after haemorrhage (Table 31) it may be concluded that hormones of adrenal origin, such as corticosteroids and catecholamines, did not significantly contribute to this proliferative response to bleeding.

Hypophysectomised rats did not exhibit increased bone marrow mitotic activity 4h after haemorrhage (Figure 46); indeed, bone marrow mitotic activity in these animals decreased in parallel with the plasma calcium concentration (Figure 47) even though the capacity of these animals to synthesize and release EPO was presumably unimpaired (Jacobson et al., 1957a). A number of hormones of pituitary origin (ADH, growth hormone, prolactin and oxytocin) have been shown to stimulate cell division in suspensions of thymic lymphocytes maintained in vitro (MacManus & Whitfield, 1969; Whitfield et al., 1969a, c; Whitfield et al., 1970a) and growth hormone is known to stimulate lymphocyte proliferation in vivo (Shrewsbury & Reinhardt, 1959). Although the doses used in the in vitro studies were probably not physiological the possibility remains that any of these, or other, pituitary hormones might have stimulated bone marrow mitosis after haemorrhage. However, the observations that injection of ADH and its analogue DDAVP stimulated bone mitotic activity at 4h (Figures 41 and 45, Table 32) strongly suggest that this neurohormone is the active mitogenic agent which produces the bone marrow response observed at 4h after haemorrhage. This conclusion is further substantiated by the observation that ADH stimulates

the rate of entry of cells into mitosis over a 4h period in bone marrow cultures <u>in vitro</u> (Figure 49). EPO, aldosterone and angiotensin were completely ineffectual in this respect. These <u>in vitro</u> observations confirm that the mitogenic activity of ADH is not mediated by the calcium homeostatic system (see Figures 44 and 48); however, the complete absence of the calcium ion in the extracellular medium has been shown to abolish the hormone's actions in thymocyte cultures (Whitfield <u>et al.</u>, 1969a). The mechanism by which ADH exerts its mitogenic action on bone marrow cells has been investigated and is reported in Chapter 5. Briefly, it seems that the mechanism is similar to that postulated on the basis of observations in <u>in vitro</u> thymocyte cultures (Whitfield <u>et al.</u>, 1969a; Whitfield <u>et al.</u>, 1970a). It certainly appears that ADH acts via the elevation of intracellular cyclic AMP concentration which presumably then initiates DNA synthesis and mitosis in the manner depicted in Figure 4 (General Introduction, Section 5.4). A more detailed discussion of the actions of ADH upon bone marrow mitosis <u>in vitro</u> is given later (Chapter 5).

Since the plasma levels of ADH 10 minutes after haemorrhage are some 30 times as great as resting levels, rapidly declining thereafter to normal, it is probable that ADH has little effect upon bone marrow mitosis under any conditions other than shortly after the onset of severe hypovolemia. Indeed, 4h after the start of CO-induced hypoxia (see above), where there is a reduction in oxygen carrying capacity but no hypovolemia, bone marrow mitosis was not significantly elevated (values not tabulated). Cellular proliferation in the bone marrow has not been measured after replacement of lost blood by homologous plasma (c.f. Figure 41). However, after loss of blood volume ADH appears to stimulate mitotic activity in the bone marrow, in addition to its classical role in the retention of body water. Haemorrhage is a powerful erythro-

poietic stimulus (c.f. Jacobson & Goldwasser, 1958; Fried et al., 1970) and the long-term elevation of bone marrow mitotic index therefore probably reflects increased mitosis within the erythroid series. However, the nature of the marrow population which is mitotically stimulated by ADH has not been established. The effect of ADH is likely to be limited to the induction of a single "wave" of division in the marrow, for the hormone is very rapidly cleared from the circulation after release (Errington & Rocha e Silva, 1971). This is borne out by the changes in marrow mitotic index which were observed over the first 6h after haemorrhage (Figure 43). If it is assumed that endogenous EPO acts similarly to the exogenously administered hormone, a quiescent period would be expected before the establishment of hypercalcaemia and increased bome marrow mitosis (see Figures 30 and 35, Chapter 2). This delay does occur after bleeding (Figures 39 and 43). Thus the novel neurohypophysial-bone marrow axis which has been demonstrated (Figures 44 - 49, Tables 31 - 33) may represent a compensatory mechanism which stimulates erythropoiesis in the short term. Since ADH has been shown to influence erythropoiesis in a single hypopituitary patient (Jepson et al. 1968) it is possible that the present observations may have significance for clinical studies.

Over a period of days the interaction of EPO with the calcium homeostatic system is probably responsible for the elevation of bone marrow cellular proliferation and the restoration of haematocrit. The present studies have not divorced the relative contributions of the two factors (EPO and extracellular calcium status), although it has previously been demonstrated (Perris et al., 1971) that aparathyroid rats require approximately twice as long as intact, pair-fed controls to restore haematocrit after blood loss. The use of anti-EPO immune serum, neuraminidase (a desialating agent — see Discussion, Chapter 2)

Figure 49, overleaf.

Figure 49: A tentative scheme for the integration of the hormonal and physicochemical responses to blood loss in the rat. Broken arrows indicate controversial mechanisms. Heavy arrows are long term mechanisms (operating for more than a few hours after haemorrhage). + = stimulatory, - = inhibitory. Within boxes, ↑= increased, ↓ = decreased. REL = release of. PV = plasma volume; BP = blood pressure; CNS = central nervous system; ADR = adrenaline; R/A = renin/ angiotensin; ALDOST = aldosterone; ADH = antidiuretic hormone; RBCs = red blood cells; Ca = calcium; PTH = parathyroid hormone; EPO = erythropoietin; BM = bone marrow.

or nephrectomy could eliminate the contribution of EPO to the erythropoietic response to haemorrhage, but all these procedures have severe limitations which will not be discussed here. However, the long term observations which have been reported above confirm the previous demonstration of the important role of the calcium homeostatic system in the mitotic response of the bone marrow to haemorrhage and suggest analogies between the actions of endogenous and exogenous EPO.

A tentative scheme is suggested in Figure 49 which attempts to explain some of the hormonal and ionic interactions which operate after severe loss of blood in the rat.

Summary.

Wistar strain rats have been shown to restore haematocrit more slowly after exsanguination than do SPF rats. The duration of the hypocalcaemic phase which immediately follows haemorrhage was also greater in the former strain.

The aetiology of the hypocalcaemia was unclear, although the dependence of this phenomenon upon the loss of red blood cells has been reaffirmed. It seems likely that a number of minor contributions resulting from plasma dilution, hypoxia and possibly changes in blood flow dynamics constitute the major decrease.

Plasma calcium concentration and bone marrow mitotic index were elevated in parallel for several days after the termination of the hypocalcaemic period. These changes have been postulated to depend upon the interaction of endogenous EPO and PTH.

A wave of increased bone marrow mitosis was observed 4h after haemorrhage. This was almost certainly produced by a sudden surge of ADH activity immediately after blood loss and not by elevated circulating levels of several other hormones. A partial scheme for the interactions of hormones and ions after haemorrhage has been presented.

CHAPTER 4.

Mechanisms of action of other agents which influence bone marrow mitosis *in vivo*.

Introduction.

Exogenous EPO (Chapter 2) and endogenous EPO released in response to haemorrhage (Chapter 3) appear to stimulate bone marrow mitotic activity via the elevation of extracellular calcium concentrations. It therefore seemed pertinent to examine the action of two other agents, cobalt and haemolysed serum, which have been claimed to increase circulating levels of endogenous EPO (Waltner & Waltner, 1929; Jacobson & Goldwasser, 1958; Erslev, 1971a; Rodgers et al., 1972; Smith & Contrera, 1972) to determine whether EPO acted via the calcium homeostatic system in these situations also.

Reductions in bone marrow mitotic rate have been demonstrated to accompany depressions in plasma calcium concentration (Rixon, 1968; Perris & Whitfield, 1971; Perris et al., 1971; Rixon & Whitfield, 1972a; Chapters 1-3). It therefore seemed valid to examine the effect of a further hypocalcaemic influence, the hormone calcitonin (CT), upon bone marrow cellular proliferation.

The cobaltous ion increases the plasma levels of endogenous EPO in normal rats within 12h (Jacobson & Goldwasser, 1958). The ion probably acts via the elevation of intracellular cyclic AMP concentrations in certain renal cells. The cyclic nucleotide is then thought to initiate increased formation of the renal erythropoietic factor (see Sections 2.7 and 2.12) which acts upon a plasma substrate to yield EPO.

Haemolysed serum has been variously postulated to stimulate erythropoiesis via the elevation of endogenous EPO release (Erslev, 1971a) or by a direct action upon bone marrow elements (Labardini et al., 1968). To attempt to distinguish between these possibilities the effect of the haemolysate could be studied both in vivo and in vitro. The action of haemolysed serum in vivo, in common with those of most other erythropoietic factors, is exaggerated in polycythemic rats in which the erythrocytic demand is negligible (Adamson & Finch, 1968).

The hypocalcaemic hormone CT rapidly reduces plasma calcium concentration in normal animals (Hirsch et al., 1964) but this action is presumably opposed and subsequently reversed by compensatory release of PTH, for both hypocalcaemia and CT are parathyrotrophic (Oldham et al. 1971). Calcitonin's action will be unopposed in PTX rats which do not produce PTH; however, the magnitude of the depression of plasma calcium concentration by CT will be reduced in aparathyroid animals because, in addition to directly stimulating bone calcium accretion, CT also acts by inhibiting bone resorption which will itself be reduced in the aparathyroid animal (General Introduction, Section 4.4). Injection of large doses of CT into PTX rats would therefore be expected to reduce plasma calcium concentration for a period of hours; plasma levels would presumably be re-established eventually since in this situation bone would be supersaturated with respect to plasma (see Introduction, Section 4.2 for a discussion of calcium homeostasis in parathyroprivic animals).

The following studies were therefore undertaken to:

(i) Determine whether endogenous EPO released by cobalt treatment could stimulate bone marrow mitotic activity and, if so, whether this action was mediated via the calcium homeostatic system.

(ii) Attempt to distinguish between those actions of red cell haemolysates upon endogenous EPO production and those directly upon bone marrow elements.

(iii) Investigate the level of bone marrow proliferative activity after CT treatment in intact and aparathyroid rats to determine whether this paralleled changes in plasma calcium concentration.

Methods.

Bone marrow mitotic activity was measured at various times after the injection of cobaltous chloride, haemolysed serum or calcitonin (CT) into intact, thyroparathyroidectomised (TPTX) or parathyroidectomised (PTX) rats. Plasma calcium concentrations were also measured after the administration of haemolysed serum and CT. The actions of haemolysed serum upon bone marrow cells maintained in vitro were also examined.

Bone marrow mitotic index and the rate of entry of nucleated cells into the arrested metaphase condition in the presence of Colcemid were determined as before (General Methodology, Section 6). Plasma total calcium concentration was measured by the titration method described previously (Methodology, Section 5.2). Blood samples were taken by cardiac puncture and plasma was prepared by centrifugation at 500xg (Methodology, Sections 4.3 and 5.1).

Cobaltous chloride, a substance which stimulates the release of endogenous EPO (Waltner & Waltner, 1929; Jacobson & Goldwasser, 1958; Rodgers et al., 1972), was dissolved in 0.9% saline and administered (1, 2.5 or 5 μmoles/100g body weight) as a single 0.5ml injection s.c. in the dorso-thoracic region of normal, TPTX and PTX rats.

Haemolysed serum was prepared from blood taken from healthy male donor rats in unheparinised syringes. Female donors were not used, for their plasma would have contained oestrogen which is an inhibitor of bone marrow cellular proliferation (Smith et al., 1974). Donor blood was allowed to clot for 15 minutes at room temperature and was then rapidly and repeatedly frozen (-20°C) and thawed (+37°C) ten times. After each thawing, tubes were violently agitated with a mechanical mixer. The haemolysed samples were centrifuged at 500xg for 15 minutes

and the serum supernatant was then drawn off and pooled. Total protein and haemoglobin measurements confirmed that this procedure had completely lysed all the red blood cells present. Rats were made polycythemic by i.p. injection of 1.5ml/100g body weight of packed red cells on two consecutive days (Day 1 and Day 2). Control rats received 1.5ml 0.9% saline. Half of the polycythemic and control animals received 2ml haemolysed serum/100g body weight and the others the same volume of normal serum i.p. on Days 5 and 6. Bone marrow mitotic index and plasma calcium concentration were then assessed in all groups on Day 8. This procedure was similar to the recommendations of Erslev (1971a) for achieving maximal circulating endogenous EPO elevation and Labardini et al. (1968) for stimulation of the maximal erythropoietic response of the bone marrow.

Some groups of rats were sham PTX, PTX and TPTX by the methods described previously (Introduction, Section 4.2).

Porcine CT (0.2 U/mg - Wilson Labs.) was dissolved in a minimum volume of 0.1M formic acid (in saline) and then diluted with 0.1M sodium acetate (also in saline). The CT solution was administered (50 MRC mU/100g body weight) s.c. as a single 0.5ml injection to sham PTX and PTX rats (0h). Colcemid (0.2mg/100g) was injected i.p. at the same time and also at 3h. Bone marrow mitotic activity and plasma calcium concentrations were assessed at 6h. Since PTX rats have severely reduced plasma calcium concentrations (c.f. Hirsch et al., 1963; Chapters 1 and 2) it was reasoned that any further reduction by the action of CT might precipitate a tetanic crisis. To prevent or ameliorate such a situation animals, after having been established to be parathyroprivic (Methodology, Section 4.2), were placed on a normal diet plus drinking water containing 1% calcium gluconate for 4 days.

Thus their calcium intake, and possibly their circulating plasma calcium levels, would be elevated. It was clear that spasmodic intake of the water containing calcium gluconate on the day of the CT administration might provide brief elevations of circulating calcium after absorption of the ion; this would be expected to stimulate waves of division in the bone marrow in a way analagous to the injection of calcium salts (Perris et al., 1967) and therefore vitiate observations of the response of the bone marrow to CT. Thus the animals were returned to a supply of normal water early on the day of the experiment.

The effects of haemolysed serum upon bone marrow cellular proliferation were also studied in an in vitro culture system (Methodology, Section 7) to ascertain whether this substance could directly influence mitotic activity in marrow cells.

Table 34 overleaf.

Treatment	% Nucleated Cells in Metaphase 6h After Colcemid		
	Day 1	Day 2	Day 3
Saline	13.3 ± 0.5 (3)	14.2 ± 0.8 (3)	13.8 ± 0.5 (3)
1 μ mole/100g Co^{++}	15.3 ± 1.2 (5)	13.6 ± 0.5 (5)	19.3 ± 0.7 (5)
2.5 μ mole/100g Co^{++}	14.9 ± 0.7 (5)	14.4 ± 1.0 (5)	17.9 ± 1.1 (5)

Table 34: Effect of cobalt upon bone marrow mitotic activity 1, 2 and 3 days after administration to rats. Cobalt was administered s.c. in 0.9% saline on Day 0. 1μ mole/100g $CoCl_2$ significantly increased the rate of entry of nucleated bone marrow cells into Colcemid metaphase ($P < 0.01$) on Day 3, as did a 2.5μ mole/100g dose ($P < 0.05$). Bone marrow mitotic activity was not significantly changed by either dose on Days 1 and 2. Numbers of animals given in parentheses.

Table 35 overleaf.

Treatment	Bone Marrow Mitotic Index at 3 Days
Normal + saline	1.4 ± 0.1 (7)
Normal + 1μ mole/100g Co^{++}	1.95 ± 0.2 (8)
Normal + 2.5μ mole/100g Co^{++}	1.85 ± 0.15 (8)
Normal + 5μ mole/100g Co^{++}	1.6 ± 0.1 (3)
TPTX + saline	0.85 ± 0.1 (5)
TPTX + 1μ mole/100g Co^{++}	0.9 ± 0.1 (5)
PTX + saline	0.95 ± 0.1 (4)
PTX + 1μ mole/100g Co^{++}	0.8 ± 0.2 (4)

Table 35: Effect of cobalt upon bone marrow mitotic index at 3 days in intact, parathyroidectomised (PTX) and thyroparathyroidectomised (TPTX) rats. 1μ mole/100g body weight $CoCl_2$ s.c. produced a significant ($P < 0.01$) increase in marrow mitotic index in intact, but not in PTX or TPTX ($P > 0.5$ in both cases) animals. 2.5μ mole/100g $CoCl_2$ also significantly increased marrow mitotic index in intact rats ($P < 0.05$) but 5μ mole/100g did not ($P > 0.1$). Values are means ± s.e.m. Numbers of animals given in parentheses.

Table 36 overleaf.

Treatment	Haematocrit	Haemoglobin (g/100ml)
Normal + serum	44.5 ± 1.0 (5)	13.5 ± 0.4 (5)
Normal + haemolysed serum	45.5 ± 1.5 (5)	13.5 ± 0.5 (5)
Polycythemic + serum	55.0 ± 1.0 (5)	17.5 ± 0.6 (5)
Polycythemic + haemolysed serum	56.5 ± 2.0 (6)	19.2 ± 0.5 (6)

Table 36: Haematocrit and blood haemoglobin concentration in normal and polycythemic rats treated with serum or haemolysed serum. See Methods for time course of injections. Polycythemic rats had significantly ($P < 0.001$) higher haematocrits and blood haemoglobin concentrations than "normal" (animals injected with saline at the same times as polycythemics received packed red blood cells). Haemolysed serum injection did not significantly affect haematocrit or blood haemoglobin in either normal or polycythemic rats ($P > 0.1$ ranging to no change). Numbers in parathenses indicate numbers of experimental animals.

Treatment	Bone Marrow Mitotic Index
Normal + serum	1.5 ± 0.1 (5)
Normal + haemolysed serum	2.0 ± 0.1 (5)
Polycythemic + serum	1.0 ± 0.1 (5)
Polycythemic + haemolysed serum	1.55 ± 0.2 (6)

Data plotted as Figure 50, overleaf. Numbers of animals given in parentheses.

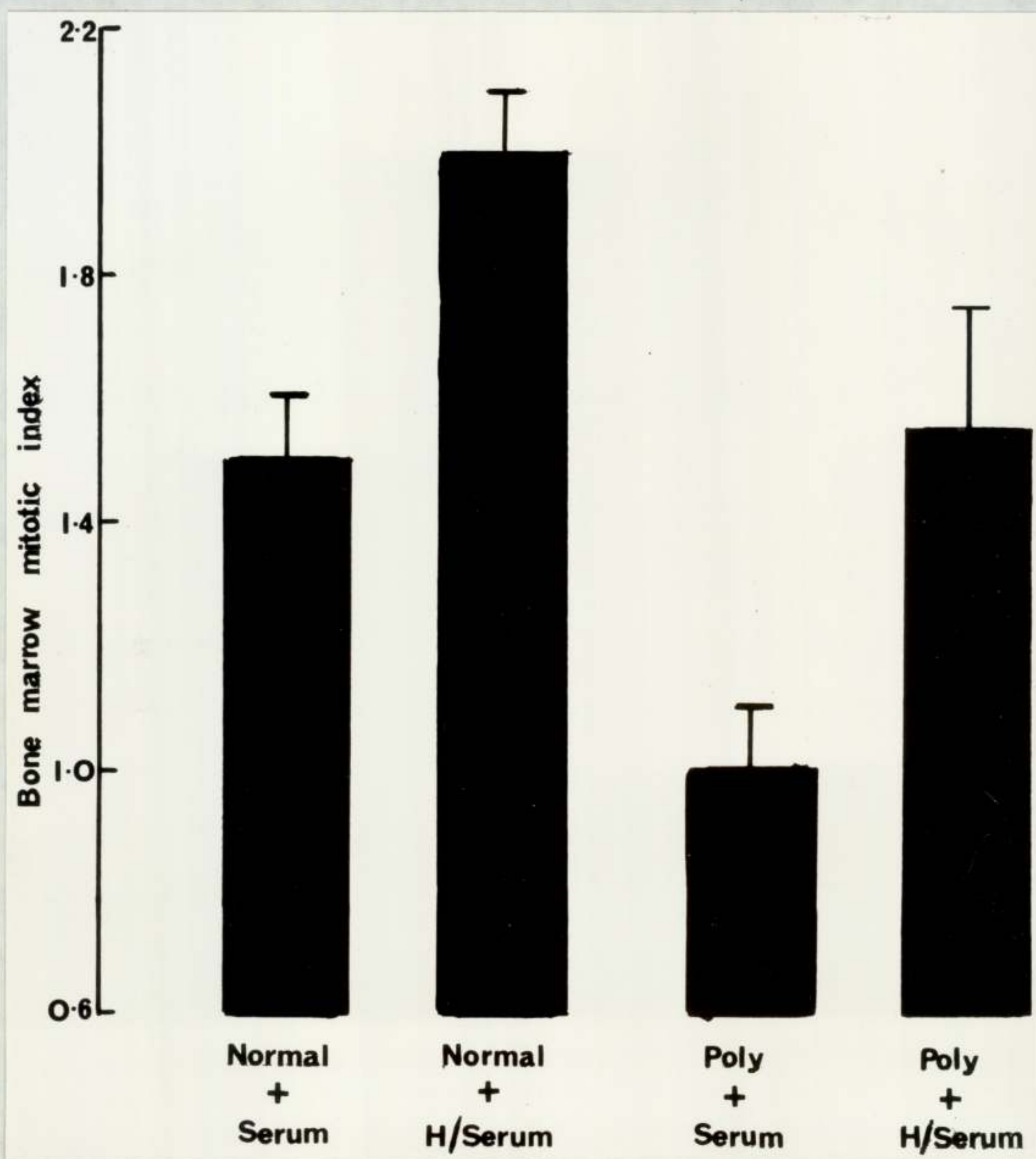


Figure 50: Effect of haemolysed serum (H/Serum) upon bone marrow mitotic index in normal and polycythemic (POLY) rats. See Methods for preparation of haemolysed serum and polycythemic rats. Haemolysed serum (2ml/100g body weight) was injected i.p. on two consecutive days. 2 days later, bone marrow mitotic index was significantly higher in normal and polycythemic rats treated with haemolysed serum than in control animals which had received normal serum ($P < 0.05$ in both cases). Columns and vertical bars represent means + s.e.m. from 5-6 animals in each case.

Results.

Bone marrow mitotic activity was significantly elevated 3 days after the administration of 1 or 2.5 µmole/100g body weight cobaltous chloride but was not increased either 1 or 2 days after injection of the ion (Table 34). This delayed response is similar to that observed after haemorrhage (Figure 43, Chapter 3). Both 1 and 2.5 µmole/100g of $CoCl_2$ significantly increased bone marrow mitotic index 3 days after injection, although 5 µmole/100g did not (Table 35). The effect of 1 µmole/100g was not apparent in aparathyroid (PTX and TPTX) rats which suggests that the ability of the animal to synthesize and release PTH is a prerequisite for the mitogenic action of cobalt. Plasma calcium concentrations were not, however, measured after the administration of cobalt.

The haematocrit and blood haemoglobin concentration of polycythemic rats were significantly higher ($P < 0.001$ in both cases) than in control (saline-injected) animals (Table 36). Administration of haemolysed serum had not further elevated blood haemoglobin level at the time of measurement (Table 36), suggesting that the exogenous haemoglobin had been sequestered or metabolised during the period intervening between the second injection and blood sampling. An alternative possibility is that the haemolysate had not been absorbed from the peritoneal cavity, but this seems unlikely because animals so treated demonstrated significantly greater bone marrow mitotic indices than animals injected with normal serum (Figure 50). This increase, which occurred in both polycythemic and normal rats, must have been stimulated by the products of haemolysis present in the injectate. It should be noted that the bone marrow mitotic index was significantly ($P < 0.01$) lower in polycythemic animals treated with normal serum than in normal animals treated identically (Figure 50). Plasma calcium concentration

Treatment	Plasma Total Calcium Concentration
Normal + serum	10.5 ± 0.2 (5)
Normal + haemolysed serum	10.2 ± 0.1 (5)
Polycythemic + serum	10.3 ± 0.1 (5)
Polycythemic + haemolysed serum	10.45± 0.2 (6)

Data plotted as Figure 51, overleaf. Numbers of animals given in parentheses.

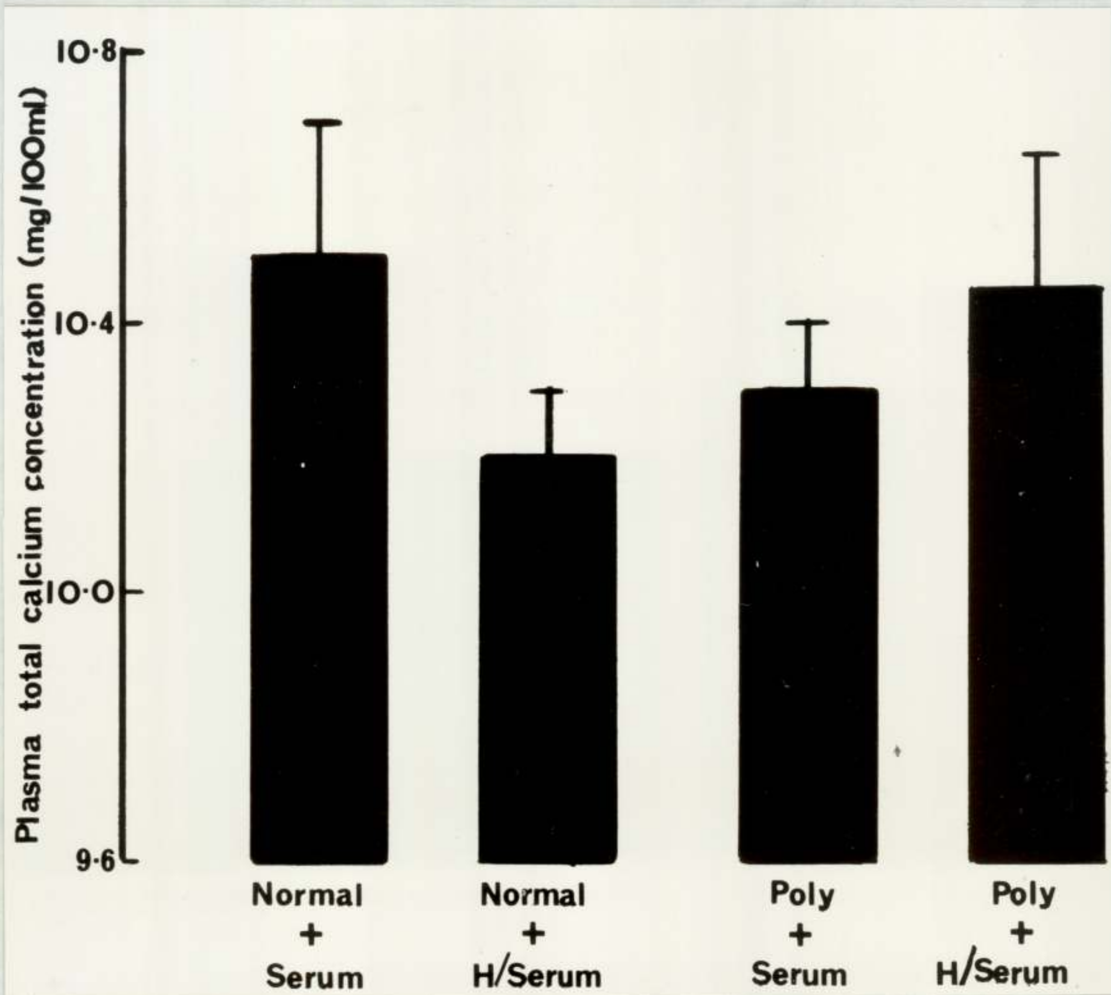


Figure 51: Lack of effect of haemolysed serum (H/Serum) upon plasma calcium concentrations in normal and polycythemic (POLY) rats. See Methods for preparation of haemolysed serum and polycythemic rats. Haemolysed serum (2ml/100g body weight) was injected i.p. on two consecutive days. 2 days later, plasma total calcium concentrations were not significantly different from those in normal serum-injected controls in either normal or polycythemic rats ($P > 0.1$ and > 0.5 respectively). Columns and vertical bars represent means ± s.e.m. from 5-6 animals in each case.

Table 37 overleaf.

Treatment	% Nucleated cells in metaphase at 6h (0.062mM Colcemid in culture)
Normal serum	4.0 ± 0.2 (4)
Normal serum + Haemolysed serum (0.15ml/ml culture)	5.1 ± 0.2 (4)

Table 37: Effect of haemolysed serum upon bone marrow mitotic activity in vitro. See General Methodology, Sections 7.3 and 7.4 for description of culture technique. Addition of 0.15ml haemolysed serum (see Methods for preparation) /ml culture significantly ($P < 0.05$) enhanced the percentage of nucleated cells which became arrested at metaphase in 6h in the presence of 0.062mM Colcemid. Numbers of duplicate cultures given in parentheses.

Table 38 overleaf.

Treatment	Plasma total calcium concentration at 6h (mg/100ml)	
sham PTX + solvent	9.7 ± 0.3 (4)	$P > 0.5$
sham PTX + CT	9.4 ± 0.2 (5)	
PTX + solvent	6.5 ± 0.2 (5)	$P < 0.01$
PTX + CT	5.6 ± 0.1 (5)	

Table 38: Effect of porcine calcitonin (CT) upon plasma total calcium concentrations in sham parathyroidectomised (sham PTX) and parathyroidectomised (PTX) rats, at 6h. See Methods for details of experimental design and dissolution of CT. The hormone was administered (50 MRC mU/100g body weight) as a single 0.5ml injection s.c. at 0h. CT significantly decreased plasma total calcium concentration in PTX, but not in sham PTX, rats. Note that PTX rats were significantly ($P < 0.001$) hypocalcaemic with respect to sham PTX animals. Numbers of animals given in parentheses.

Table 39 overleaf.

Treatment	% Nucleated bone marrow cells in metaphase 6h after Colcemid	
Sham PTX + solvent	13.3 ± 0.7 (4)	P > 0.1
Sham PTX + CT	10.9 ± 1.1 (5)	
PTX + solvent	20.9 ± 0.7 (5)	P < 0.001
PTX + CT	16.1 ± 0.5 (5)	

Table 39: Effect of porcine calcitonin (CT) upon bone marrow mitotic activity at 6h in sham-parathyroidectomised (Sham-PTX) and parathyroidectomised (PTX) rats. See methods for details of maintenance of animals and dissolution of CT. The hormone was administered (50 MRC mU/100g body weight) as a single 0.5ml injection s.c. at 0h. Colcemid (0.2mg/100g body weight) was administered i.p. simultaneously and again a 3h. CT significantly reduced the percentage of nucleated cells which reached metaphase in 6h in PTX rats but did not do so in sham operated animals. Note that mitotic activity was significantly ($P < 0.001$) higher in control PTX rats than in sham PTX animals; See Discussion. Numbers of animals given in parentheses.

was slightly, but not significantly, lower also (Figure 51). This point is considered further in the Discussion.

Although EPO has been demonstrated to elevate plasma calcium concentrations (Chapters 2 and 3) haemolysed serum did not do so in either normal or polycythemic animals (Figure 51). This suggested that EPO might not have been released by haemolysed serum treatment in these experiments. Certainly, haemolysed serum was capable of directly stimulating bone marrow mitotic activity in vitro (Table 37).

Six hours after calcitonin injection plasma calcium concentrations were significantly lowered ($P < 0.01$) in PTX, but not in sham-PTX, rats (Table 38). The rate of accumulation of nucleated bone marrow cells in Colcemid metaphase over the 6h test period was reduced by CT (Table 39) in parallel with plasma calcium concentration in PTX rats ($P < 0.001$); CT did not significantly affect marrow mitotic activity in sham PTX animals (Table 39). The surprisingly high rate of progression of marrow cells into metaphase in the PTX rats is considered later (Discussion).

Discussion.

The observations of bone marrow proliferative activity after cobalt injection are incomplete. They do, however, provide a preliminary indication that this action of cobalt might be dependent upon the presence of the parathyroids (see Table 35). Since the ion stimulates erythropoiesis via the elevation of circulating EPO (Jacobson & Goldwasser, 1958; Rodgers et al., 1972; General Introduction, Section 2.12) the present observations are consistent with the hypothesis which has been developed for the mechanism of action of EPO upon bone marrow mitosis (Chapters 2 and 3), i.e. that the hormone acts via the elevation of extracellular calcium concentration, the ionised form of which is the primary mitotic stimulant. Unfortunately plasma calcium concentrations have not been measured after cobalt treatment. These measurements are required to definitely establish whether this is a further situation in which endogenous EPO acts via modulations of the calcium homeostatic system. It appears that a direct action of cobalt upon bone marrow, which has been reported (Fisher et al., 1964) to increase nucleated erythroid cell count in dogs, is not of significance in the present situation for no mitotic stimulation was observed in aparathyroid rats (Table 35).

The delayed appearance of the stimulation of marrow proliferation 3 days after cobalt injection (Table 34) is similar to that observed after haemorrhage (Chapter 3). This phenomenon may be explained by the demonstration of a prolonged hypocalcaemic phase, again analogous to that observed after haemorrhage, occurring after cobalt treatment (Perris, personal communication). This could be a result of the histotoxic hypoxia induced by cobalt (Rodgers et al., 1972) which might allow the movement of calcium from plasma into the tissues (see Perris

et al., 1971; Discussion, Chapter 3 for rationale). However, it should be recalled that carbon monoxide-induced hypoxia did not induce a significant hypocalcaemia (Table 30, Chapter 3). Possibly cobalt directly inhibits the active resorption of calcium from bone by the PTH/vitamin D system (Introduction, Sections 4.3 and 4.5).

Haemolysates have been reported to stimulate erythropoiesis by a direct action upon marrow elements (Labardini *et al.*, 1968) and by promoting the release of endogenous EPO (1971a). In the present study, haemolysed serum increased bone marrow mitosis both *in vivo* and *in vitro* (Figure 50, Table 37). The *in vivo* increase in mitotic index was greater in percentage terms in polycythemic rats than in normal animals, although the absolute increases in the proportion of the nucleated marrow cells undergoing mitosis were similar (Figure 50). Plasma calcium concentration was not influenced by the administration of haemolysed serum (Figure 51). Thus one must postulate either that the mode of action of endogenous EPO in this situation is different to that observed after haemorrhage (Chapter 3) and distinct from that of the exogenously administered hormone (Chapter 2) or that haemolysed serum did not provoke EPO release in the present experiment. In this context it is interesting to note that thymocyte proliferation is elevated by a variety of stimuli which are mediated via circulating EPO but not by haemolysed serum administration (Chapter 6).

Although the data obtained by Erslev (1971a) appear consistent, the experimental design which he reports is poor in a number of ways:
(i) Control animals did not receive any injections at the same time that packed red cells were administered to other rats to produce polycythemia. Thus any effect of stress would only be apparent in polycythemic rats.
(ii) Haemolysates were prepared by suspending red blood cells in water

followed by freezing and thawing. Thus the product would be hypotonic when injected into the experimental rats. Furthermore, red cell debris was not separated from the suspension and was therefore also injected into the abdominal cavities of the recipients. Note that red cell stroma and protein mixtures do not stimulate erythropoiesis (Labardini et al., 1968; see also Figure 29, Chapter 3).

(iii) Controls for the haemolysate-injected rats received physiological saline rather than normal, homologous serum. This would appear to be an elementary oversight, for under normal conditions plasma contains active EPO (c.f. Gordon & Zanjani, 1970), albeit at low concentrations.

Although it is not obvious that any of these shortcomings could vitiate the observations of this worker, his results appear to have some inconsistencies. For instance, rat and mouse haemolysates were both capable of stimulating erythropoiesis in polycythemic rats but not in hypertransfused mice. Human and rabbit preparations were also ineffective in either rodent. This is difficult to understand, for the pattern of erythropoietic control is very consistent throughout mammalian species.

It is obviously desirable that the actions of haemolysed serum should be investigated in TPTX and PTX rats for if the agent was then still effective in stimulating bone marrow mitosis it would be clear that its erythropoietic effects were not mediated via modulations of the calcium homeostatic hormones. It would also be pertinent to study the effects of haemolysates in nephrectomised rats which do not produce significant quantities of EPO (Jacobson et al., 1957b; Introduction, Section 2.6). The physiological significance of red cell breakdown products as stimulants of erythrocyte formation is probably negligible. It may be calculated that approximately 0.05ml/100g body weight of red

blood cells (i.e. 5×10^8 cells) are destroyed each day, whereas the injections of haemolysed serum which were administered each contained the contents of 2ml/100g body weight of red cells (i.e. 2×10^{10} cells). Thus each of the two injections contained haemolysis products equivalent to those produced in 40 days of normal red cell attrition. This system could, however, possibly be of significance in some haemolytic syndromes.

The mitotic index of the bone marrow of polycythemic rats was significantly ($P < 0.001$) less than that of their controls (Figure 50) although the ambient plasma calcium concentrations of the hypertransfused animals were not significantly lower than in normals (Figure 51). In polycythemic animals the circulating levels of EPO are negligible (Adamson & Finch, 1968) and thus this lack of available EPO was presumably the cause of the lowering of the proportion of bone marrow cells undergoing mitosis. Since EPO does not appear to demonstrably stimulate marrow proliferation by a direct, as opposed to a calcium-mediated, action (Figure 32 and Table 33, Chapter 3) this was presumably a result of the removal of EPO's differentiating action upon committed stem cells (ERCs - see Introduction, Sections 2.10 and 2.11). There was no erythrocytic demand, and therefore EPO release, for 5 days prior to the time at which bone marrow mitotic index was measured (see Methods). Over this period the early erythroblasts would have become depleted by division and maturation to yield increasingly mature, non-dividing, nucleated erythroid elements. Since there would be no replenishment of erythroblasts from the ERC population in the absence of EPO the number of dividing cells in the erythroid series would decrease more rapidly than the number of nucleated cells; the marrow mitotic index (the percentage of nucleated cells in any stage of mitosis from late prophase to the end of telophase) would therefore be reduced. The

mitotic index would also probably be reduced if all the erythroblasts had matured to the erythrocyte (non-nucleated) stage, for the mitotic activity of the other stem cell compartments is probably lower than that of the erythroid series (c.f. Lajtha, 1970). These considerations indicate that the observation of suppressed marrow mitotic index in conditions of unchanged plasma calcium, but negligible plasma EPO, concentrations does not vitiate the concept that EPO exerts its mitogenic activity via the calcium homeostatic system.

The reduction in plasma calcium concentration induced by CT in PTX rats (Table 38) was accompanied by a highly significant reduction in the rate of entry of nucleated bone marrow cells into the arrested metaphase condition (Table 39). Presumably these two factors were causally related, for CT does not directly reduce bone marrow mitosis _in vitro_ (Figure 52, Chapter 5). In intact rats the hormone induced slight, but insignificant, decreases in these parameters; presumably release of PTH had compensated for the decrease in plasma calcium concentration (Introduction, Section 4.3). Two particularly striking phenomena in this experiment were the low plasma calcium level and the high rate of mitotic activity in the bone marrow of the PTX animals.

The PTX rats had been maintained on a normal diet plus 1% calcium gluconate in the drinking water for 4 days prior to the experiment (see Methods). During this period their outward appearance and activity improved, which suggested that they had increased their calcium intake and to, some extent, reversed the hypocalcaemia which they had exhibited after the cautery of the parathyroid glands. On the day of the administration of CT they had been returned to normal drinking water to prevent irregular intake of ionised calcium. Thus plasma calcium concentrations might have decreased during the day to attain the final level (in control PTX animals) of 6.5 $\pm$ 0.2 mg/100ml which

was only slightly higher than the value which had been observed when the animals were eating a calcium-deficient diet and drinking distilled water. This could only be confirmed by further experimentation.

The high level of marrow mitotic activity which was observed in the PTX rats (approximately 60% higher than in sham-PTX controls) was most unexpected for it is well established that removal of the parathyroid glands depresses bone marrow proliferation (Rixon, 1968; Perris & Whitfield, 1971; Perris et al., 1971; Rixon & Whitfield, 1972a; Chapters 1 - 3 and Table 35, this Chapter). A number of explanations must be considered;

(i) It is possible that the CT solvent, a mixture of formic acid and sodium acetate, was mitogenic. It would be necessary to further postulate that this action was not evident in the presence of the parathyroid glands for the marrow mitotic activity of the control sham-PTX rats was similar to that which has been observed in normal animals (Perris & Whitfield, 1971; Chapter 2). This seems unlikely because the solvent does not increase bone marrow (Chapter 5) or thymocyte (Morgan, personal communication) mitosis in vitro.

(ii) The parathyroid glands might have been incompletely cauterised or the animals could have possessed accessary parathyroid tissue. In either situation hyperplasia of the remaining parathyroid tissue might have re-established the capacity of the animal to secrete PTH in response to falling plasma calcium concentrations (which might have occurred during the day — see above). PTH is directly mitogenic in high concentrations in thymocytes (Whitfield et al., 1970b) and possibly bone marrow cells (personal, unpublished, observations) in vitro. However, if circulating PTH levels had been dramatically elevated it is inconceivable that plasma calcium levels would not have been increased

also (see Table 38).

(iii) Plasma calcium concentrations might have been high at the beginning of the day (just after the period of maximum water, and therefore calcium gluconate, intake — Besch, 1970) and triggered the DNA synthetic and mitotic events in a large number of bone marrow cells which subsequently accumulated at metaphase. However, sham PTX rats had also received a calcium supplement in their drinking water and the proliferation in their bone marrow tissue was at a normal level (Table 39).

(iv) Suppression of the mitotic activity of the erythroid and lymphoid bone marrow compartments by the depression of extracellular calcium concentration (c.f. Rixon & Whitfield, 1972a) might have allowed an increase in proliferation of the granuloid series (which appears to be independent of calcium ion status - Rixon & Whitfield, 1972a) by reducing "stem cell competition" (c.f. Hellmann and Grate, 1967; see also Discussion, Chapter 3). It seems unlikely that the large elevation in bone marrow mitosis which was evident (Table 39) could be solely a result of increased granulopoiesis.

(v) Differences in experimental conditions, save those resulting from the disparate endocrinological status of the animals, can probably be eliminated.

(vi) If there was a very rapid turnover of bone calcium (somehow resulting from the sudden restriction of the calcium intake) it might influence cell division in the bone marrow which is of course surrounded by the areas of calcium exchange. There is, however, no evidence that this change in plasma/bone kinetics could occur or that it could affect bone marrow proliferation.

(vii) The animals had been parathyroprivic for 9 days prior to the administration of CT, in comparison to the more usual 4-5 days (e.g. Chapter 2). It is possible that a compensatory increase in the synthesis

of active vitamin D metabolites might have occurred (Introduction, Section 4.5). The mitogenicity of these metabolites is unknown. Rixon & Whitfield (1972a) have observed that plasma calcium concentration and bone marrow mitotic activity rose towards normal in PTX rats maintained for 8 days on a calcium-containing diet. However, they did not observe an "overshoot" in marrow cellular proliferation and neither was the ambient plasma calcium concentration as low as in the present study.

None of the above hypotheses completely accounts for the observations (Table 39) and thus further experimentation is required. Monitoring the changes in marrow mitotic activity and plasma calcium over the post-operative period would help to establish the nature of the stimulus for the supranormal proliferation. This would be highly desirable, for this is one of the few situations which have been encountered in which there is a significant divergence between extracellular calcium status and bone marrow mitotic rate (Perris, 1971; Whitfield et al., 1973c; Introduction, Sections 5.2 and 5.6; Chapters 1-3).

The observations reported in this chapter are generally unsatisfactory for the experimental evidence is incomplete. However, it has been established that the action of cobalt upon bone marrow mitosis at 3 days is probably dependent upon the presence of the parathyroid gland. In addition CT has been shown to depress bone marrow mitosis in PTX animals, probably via the reduction of plasma calcium concentration. The interesting possibility has also been raised that injection of haemolysates does not stimulate endogenous EPO production. The significance of all these observations awaits confirmation by further experimentation but they largely fit with the hypotheses which have been propounded for the mechanism of EPO's actions upon bone marrow mitosis

and for the importance of the extracellular calcium concentration in the determination of the level of marrow cell proliferation.

Summary.

Cobaltous chloride increased mitotic activity in the bone marrow 3 days after administration. This action was not evident in aparathyroid animals.

Haemolysed serum elevated bone marrow proliferation both *in vivo* and *in vitro*. It did not, however, elevate plasma calcium concentration.

CT decreased plasma calcium concentration in PTX rats and thereby, it was concluded, produced the depression in bone marrow mitotic rate which was observed.

CHAPTER 5.

Mechanisms of the mitogenic actions of the calcium ion and anti-diuretic hormone in bone marrow cultures.

Introduction:

Parallels between bone marrow mitotic activity and the extracellular ionised calcium concentration have been established in a number of in vivo and in vitro situations (see General Introduction, Section 5; also Chapters 1-4). Similar patterns have been established for thymocyte proliferation (Introduction, Section 5; Chapter 6) and the ease of thymocyte cell culture has facilitated a number of investigations which have suggested a tentative mechanism for the mitogenic action of the calcium ion upon thymic lymphoblasts (Introduction, Section 5.4).

Because of the similarities between the responses of bone marrow cells and thymic lymphoblasts to elevations of extracellular calcium concentration (c.f. Introduction, Sections 5.2 and 5.3) it was pertinent to attempt to elucidate the mechanism of the mitogenic action of the calcium ion in in vitro bone marrow cultures. In addition, the mode of action of anti-diuretic hormone (ADH), which stimulates bone marrow cellular proliferation both in vivo and in vitro (Chapter 3), remained to be established.

The mitogenic action of the calcium ion in suspensions of thymic lymphocytes maintained in vitro is mediated via the elevation of the intramembranal concentration of cyclic adenosine 3', 5'-monophosphate (cyclic AMP - Whitfield et al., 1973c; see also Introduction, Section 5.4 and Figure 4). The observations that exogenous cyclic AMP stimulated mitotic activity in bone marrow cells in vivo and thymocytes both in vivo and in vitro (MacManus & Whitfield, 1969a; Rixon et al. 1970) suggested that endogenous cyclic AMP might also be implicated as a mitogenic initiator in isolated bone marrow cells. To investigate this possibility, three agents which impinge upon cyclic AMP metabolism, and which might therefore be expected to influence mitotic activity,

were utilised. Imidazole stimulates the action of a membrane-bound phosphodiesterase (PDE) which degrades cyclic AMP to adenosine 5'-monophosphate (5'-AMP), thus reducing cyclic AMP levels. Conversely caffeine inhibits the actions of PDE (Robison et al., 1971) and will thus increase endogenous cyclic AMP concentrations. The hormone calcitonin (CT) has been shown to antagonise the mitogenic activities of ADH, parathyroid hormone (PTH) and extracellular cyclic AMP in thymocyte cultures (MacManus & Whitfield, 1970; Whitfield et al. 1970a). The action of CT is thought to be similar to that of imidazole, i.e. to potentiate PDE activity (MacManus & Whitfield, 1970; Whitfield et al. 1970a) and so decrease intramembranal cyclic AMP concentration. Thus CT is also a useful tool for the indication of the involvement of cyclic AMP in mitogen-cell interactions.

Although Morton has demonstrated that elevated calcium ion concentrations stimulated mitotic activity in bone marrow cell cultures (Morton, 1968; Morton et al., 1968) the precise concentration of the ion in the culture medium was not measured.

The present studies were undertaken to:

(i) Determine the level of mitotic activity in bone marrow cells in vitro at known concentrations of ionised calcium; and

(ii) Investigate the possible involvement of c-AMP in the mitogenic actions of the calcium ion and ADH in bone marrow cultures.

Methods.

The effects of several agents upon bone marrow mitotic activity were investigated in the *in vitro* culture system which has been described and discussed previously (General Methodology, Section 7.3 and 7.4). Bone marrow cells from male Wistar rats, weighing 90 - 110g, were incubated at 37°C for 4-6h in 100% rat serum in a humidified atmosphere of 30% CO_2/70% air. The metaphase-arresting agent Colcemid (Methodology, Section 6.4) was dissolved directly in the serum to give a final concentration of 0.062mM. All cultures were performed in duplicate.

The ionised calcium concentration of the culture medium was normally adjusted to 1.2mM (approximately the level in normal serum), or in some cases to 1.8 or 2.4mM, by addition of small volumes of a $CaCl_2$ solution (Methodology, Section 7.4). All solutions were made up in 0.9% saline. Hormones and other agents were dissolved directly in the serum wherever possible or were added as small (10μl) volumes in saline solution. Imidazole and caffeine were added as saline solutions to some cultures containing 1.8mM ionised calcium or 0.6mU/ml ADH to give final concentrations of 0.5mM and 0.4mM respectively. However CT (Wilson Laboratories, activity 0.2U/mg, final concentration in culture 8 MRC mU/ml) was first dissolved in 0.1M formic acid and made up to volume with 0.1M sodium acetate. Control cultures for the CT studies contained the same concentrations of formic acid and sodium acetate.

ADH (Parke-Davis) was added directly to the cultures to give a final concentration of 0.6mU/ml, which is equivalent to the circulating levels observed a few minutes after haemorrhage (see Chapter 3 for discussion).

At the end of the culture period the cell suspensions were concentrated (Methodology, Sections 7.3 and 7.4) and were then smeared, dried and stained as before (Methodology, Section 6.2). Smears were scored for the percentage of nucleated cells in metaphase (Methodology,

Sections 6.4 and 6.5). At least 1500 cells per culture were counted, and the means of the two results from the duplicate cultures were used as a single experimental determination.

Table 40 overleaf.

Ionised calcium concentration (mM)	% Nucleated cells in metaphase at 6h (0.062mM Colcemid present in medium)
1.2	4.0 ± 0.2 (6)
1.8	5.4 ± 0.3 (6)
2.4	5.3 ± 0.4 (4)

Table 40: Effect of elevated ionised calcium concentration upon bone marrow mitotic activity *in vitro*. See General Methodology, Sections 7.3 and 7.4 for description of culture technique. Elevation of the ionised calcium concentration of the culture medium to 1.8mM and 2.4 mM significantly increased the percentage of nucleated bone marrow cells which reached metaphase in 6h ($P < 0.01$ and $P < 0.05$ respectively). Numbers of cultures given in parentheses.

Treatment	% Nucleated cells in metaphase at 6h (Colcemid present)
1.2mM Ca^{++}	4.0 ± 0.2 (4)
1.8mM Ca^{++}	5.1 ± 0.2 (4)
1.8mM Ca^{++} + IMID	4.0 ± 0.1 (4)
1.8mM Ca^{++} + CAFF	5.7 ± 0.3 (4)
1.8mM Ca^{++} + CT	3.4 ± 0.3 (4)

Data plotted as Figure 52, overleaf.

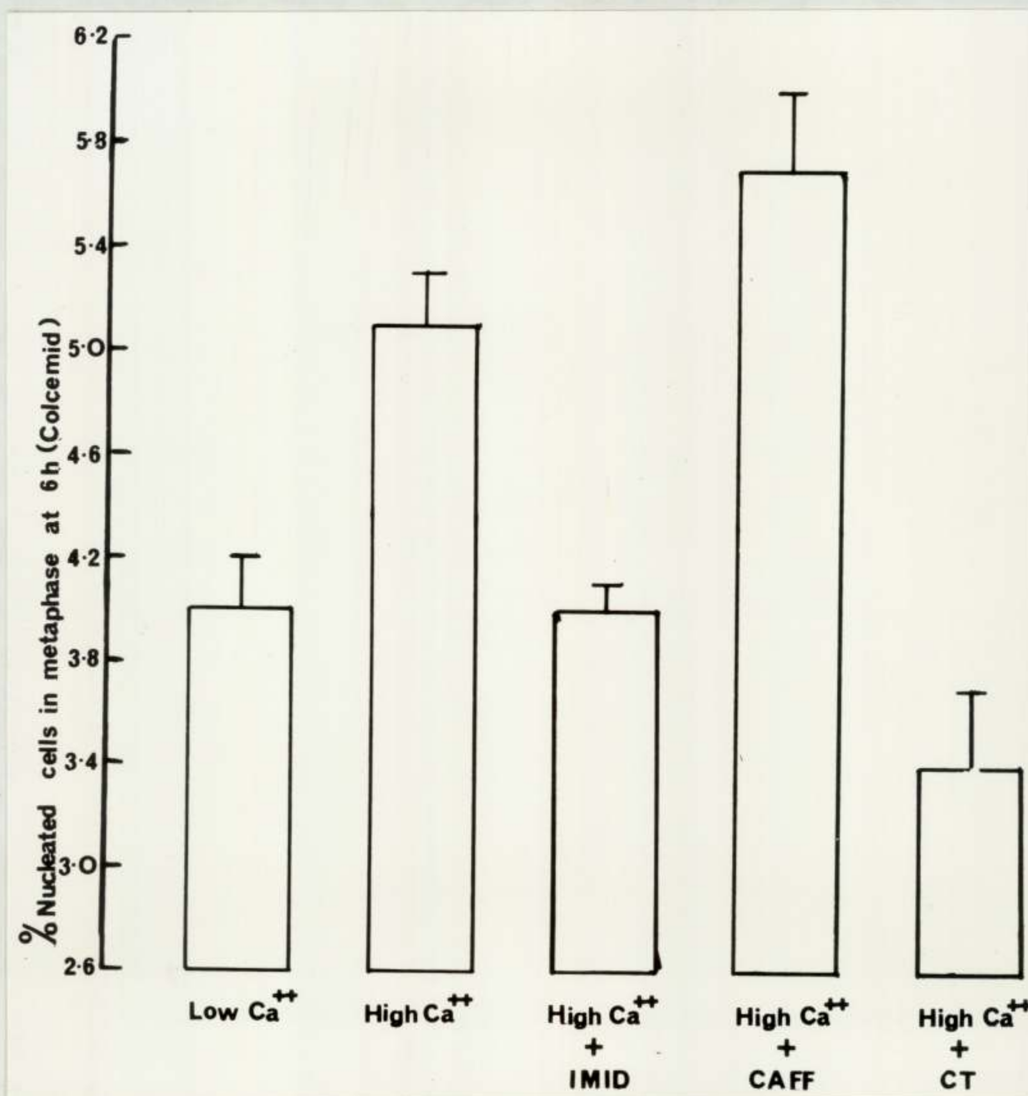


Figure 52: Effects of imidazole (IMID), caffeine (CAFF) and calcitonin (CT) upon the elevation of bone marrow mitosis *in vitro* induced by increased levels of extracellular calcium. See General Methodology, Sections 7.3 and 7.4 for details of culture technique. Increasing the ionised calcium concentration of the medium from 1.2mM (Low Ca^{++}) to 1.8mM (High Ca^{++}) significantly ($P < 0.05$) increased the percentage of nucleated cells which reached metaphase at 6h in the presence of 0.062mM Colcemid. Imidazole and CT completely abolished this increment ($P > 0.5$) and caffeine potentiated it, although this potentiation was not significant ($P > 0.1$). Columns and vertical bars represent mean ± s.e.m. from 4 duplicate cultures in each case.

Treatment	% Nucleated cells in metaphase at 4h (0.062mM Colcemid present)
Control	2.8 ± 0.1 (6)
ADH (0.6mU/ml)	4.0 ± 0.2 (6)
ADH + imidazole (0.5mM)	3.1 ± 0.1 (4)
ADH + caffeine (0.4mM)	5.0 ± 0.1 (4)

Data plotted as Figure 53 overleaf. Numbers of cultures in parentheses.

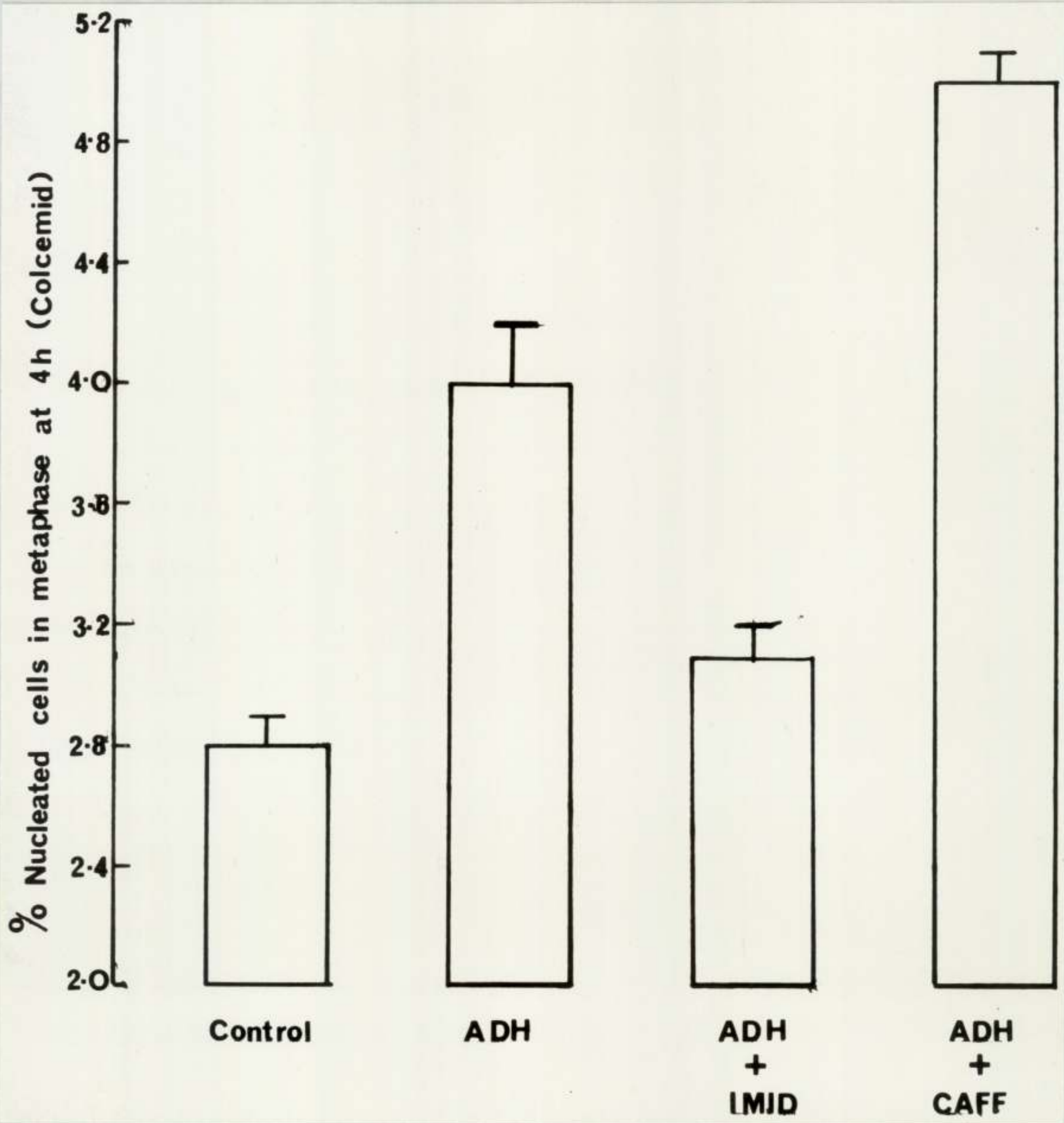


Figure 53: Effects of imidazole (IMID) and caffeine (CAFF) upon anti-diuretic hormone (ADH)-induced mitotic activity in bone marrow cells in vitro. See General Methodology, Sections 7.3 and 7.4 for description of culture technique. ADH (0.6mU/ml) significantly ($P < 0.01$) increased the number of nucleated cells which reached metaphase in 4h in the presence of 0.062mM Colcemid. This action was completely abolished by imidazole (0.5mM) but was significantly ($P < 0.05$) potentiated by caffeine (0.4mM). Columns and vertical bars represent means + s.e.m. from 4-6 cultures in each case.

Results.

Elevation of the extracellular calcium ion concentration from the basal level of 1.2mM to 1.8 or 2.4mM significantly increased the percentage of nucleated bone marrow cells which reached metaphase in 6h of culture (Table 40), confirming previous in vitro observations in thymocytes (Whitfield et al., 1969b) and bone marrow cells (Morton, 1968; Morton et al., 1968).

The mitogenic activity of elevated ionised calcium concentrations was abolished by the addition of 0.5mM imidazole and 8mU/ml CT to the medium (Figure 52). However, addition of 0.4mM caffeine potentiated the increase in bone marrow cellular proliferation produced by the elevation of the calcium content of the medium (Figure 52), although this potentiation was not significant (possibly because of the small numbers of cultures which were performed). Since imidazole and CT increase the intracellular degradation of cyclic AMP, while caffeine decreases this process, the effects of these agents in the present system would suggest that calcium exerts its mitogenic action via the elevation of intracellular cyclic AMP.

Anti-diuretic hormone significantly ($P < 0.01$) increased the percentage of nucleated bone marrow cells which became arrested at metaphase in 4h in the presence of Colcemid (Figure 53), thus paralleling its activity in vivo (Chapter 3).

The in vitro increase was also abolished by 0.5mM imidazole and significantly ($P < 0.05$) potentiated by 0.4mM caffeine, thus inferring once again that cyclic AMP might be invovlved as a mediator of the mitogenic action of a hormone.

Discussion.

The present observations closely resemble those which have established a working hypothesis for the mitogenic actions of elevated extracellular ionised calcium concentration and ADH in suspensions of thymic lymphocytes maintained in vitro (Whitfield et al., 1969b, 1970a; MacManus & Whitfield, 1971). Although measurements of intracellular cyclic AMP concentration have not been made in the bone marrow cultures, the results strongly suggest that this cyclic nucleotide mediates the mitogenic actions of ionised calcium and ADH (Figures 52 and 53).

Since mitotic stimulation by elevated extracellular calcium concentrations in bone marrow cells both in vivo and in vitro is followed by increased reticulocyte production (Morton, 1968; Perris & Whitfield, 1971) it is probably that the parallel observations upon the mitogenicity of calcium made here (Table 40) also represent, at least in part, increased erythropoietic activity. It is hoped that these observations may be repeated in the future with concomitant measurement of radioiron incorporation into the bone marrow elements to provide a further index of erythropoiesis in the presence of heightened calcium concentrations. On the basis of the observations made here (Table 40, Figure 52) it is tempting to postulate that agents which affect bone marrow cellular proliferation via the elevation of mobile calcium, such as EPO (Chapter 2), do so by the cyclic AMP-dependent mechanism which has been inferred in these studies. However, it is not clear that the observations reported above have significance for the in vivo situation. Certainly exogenous cyclic AMP stimulates marrow mitotic activity in vivo (Rixon et al., 1970), as it does in thymic lymphoblasts both in vivo and in vitro (MacManus & Whitfield, 1969a; Rixon et al., 1970). Since the cyclic AMP molecule does not enter thymocytes to exert its mitogenic action (MacManus et al., 1971b) it is not certain that the parallel responses

to extracellular nucleotide which have been shown in the two tissues are necessarily related to its role as an intracellular second messenger for mitogenic agents. It therefore seems that measurements of tissue levels of cyclic AMP after the application of the mitotic stimulus of increased extracellular calcium concentration are required to determine whether the nucleotide is as vital for the in vivo activity of the ion as it appears to be in vitro. Certainly, the consistency of the in vitro observations and the roles which have been demonstrated for cyclic AMP in the mediation of the mitogenic actions of a wide range of biogenic agents in thymocyte culture (MacManus & Whitfield, 1969, 1971; Whitfield et al., 1970a, b, c; Morgan, unpublished) make untenable the possibility that the mitogenic mechanisms which occur in thymocyte suspensions are artefacts of the in vitro situation. It seems likely that the mechanism of action which has been postulated for the calcium ion (General Introduction, Section 5.4; Figure 4; also see below) has significance for the whole animal because many parallels have been demonstrated between the mitogenic responses of thymic lymphoblasts to the ion in vivo and in vitro (General Introduction, Sections 5.2, 5.3 and 5.5).

The evidence presented previously (Chapter 3) has strongly suggested that the increase in bone marrow mitotic activity which occurs 4h after haemorrhage is directly triggered by the rapid elevation of circulating ADH which occurs within a few minutes of blood loss. This in vivo mechanism was not mediated via the parathyroid gland or the elevation of extracellular calcium concentration (Figures 44 and 48, Chapter 3). The present observations (Figure 53) suggest that ADH directly stimulates mitotic activity in bone marrow cells by a mechanism dependent upon cyclic AMP mediation, for imidazole inhibits the mitogenic action of the hormone while caffeine significantly potentiates it. The mitogenicity of ADH upon thymocytes has been demonstrated to be dependent upon the presence of the calcium ion in the culture medium (Whitfield et al., 1969a).

It is not possible to completely remove calcium from the serum medium used for bone marrow culture without the use of chelating agents which might also bind cellular calcium, thus probably reducing cell viability and interfering with the mitotic process. The dependence of ADH upon the presence of the calcium ion has therefore not been demonstrated for bone marrow cells.

ADH has been postulated to act upon thymic lymphoblasts which are poised at the G_1/S boundary (see Figure 5) by increasing membrane permeability to the calcium ion which constitutes the primary mitogenic stimulant (Whitfield et al., 1969a). If this mechanism also operates in bone marrow cells it is necessary to envisage a very short ($<$ 4h) period for the progression of recruited cells through the S and G_2 phases into mitosis, for ADH has been demonstrated to significantly elevate marrow mitotic activity both in vivo (Chapter 3) and in vitro Figure 53) within 4h.

Although the present observations are incomplete without measurements of cyclic AMP concentration and DNA synthesis they do infer that both the calcium ion and ADH exert their mitogenic actions via the elevation of intracellular cyclic AMP concentration. The increased levels of cyclic AMP, which are probably most evident within the cell membrane (Whitfield et al., 1973c), are then envisaged to act via the release of initiating factors from membrane activation sites which somehow provoke initiation of DNA synthesis and mitosis as discussed previously (General Introduction, Section 5.4; Figure 4).

Summary.

Increased extracellular concentrations of the calcium ion stimulated mitotic activity in bone marrow cells maintained *in vitro* for 6h. This action was inhibited by imidazole and CT and potentiated by caffeine, suggesting that the mitogenic activity of the ion was mediated via the elevation of intracellular cyclic AMP concentration.

ADH, in a concentration equivalent to that observed in the circulation after haemorrhage, also stimulated bone marrow cellular proliferation *in vitro*. Again, it was suggested that this action was mediated via endogenous cyclic AMP, for imidazole inhibited the mitogenic action of the hormone while caffeine potentiated it.

The significance of these observations for the whole animal has not been established, though studies in other systems by other workers suggest that similar mechanisms may operate *in vivo*.

CHAPTER 6.

Thymocyte proliferation during periods of enhanced bone marrow mitotic activity.

Introduction.

An increasing body of evidence has accumulated which suggests that the thymus, in addition to its role in the development of immunocompetence (General Introduction, Section 3.3), may also influence haemopoiesis. Since there are striking parallels between the proliferative responses of the thymus and bone marrow to various procedures which re-adjust the ambient excellular ionised calcium concentration (Perris, 1971; Whitfield et al., 1973c; Introduction, Sections 5.2, 5.3, 5.5 and 5.6; Table 1; Chapter 1; Discussion, Chapter 5) it seemed worthwhile to examine the patterns of thymic mitotic activity after various erythropoietic stimuli. In addition, the mitogenic activity of anti-diuretic hormone (which has been observed in bone marrow — Chapters 3 and 5) required examination as a possible biogenic stimulant of thymocyte proliferation.

Thymectomised rats and foetal opossums have greater proportions of immature erythroblast cells in their bone marrow than do normal animals (Miller, Block, Rowlands & Kind, 1965; Corsi & Giusti, 1967). In addition, anaemia has been reported following neonatal thymectomy in mice (Metcalf, 1966b) and adult thymectomy in this species reduced the number of endogenous colony forming units (CFUs - see Introduction, Section 2.10) in the bone marrow (Hrsak, 1973). Enhancement of murine spleen growth in vivo (Metcalf, 1964) and in vitro (Auerbach, 1963; Metcalf, 1968) by the thymus have also been observed. It should be noted that the spleen is an erythropoietic organ in the mouse well into adult life (Fruhman, 1970; Bozzini, Martinez, Alippi & Chait, 1972).

In contrast to the above observations, which suggest that the presence of the thymus enhances haemopoiesis, human thymomas have been reported to induce erythroid aplasia, probably because of the

excessive production of an erythropoietic inhibitor (Al-Mondhiry, Zanjani, Spivack, Zalusky & Gordon, 1971).

A series of observations have indicated that thymocytes themselves are the thymus constituent which may influence haemopoiesis in situations such as those described above. Thus, the poor growth of transplanted parental (P_1) marrow cells in irradiated hybrid (P_1 x P_2) F_1 mice was augmented by thymocytes isogeneic with the parental marrow (Goodman & Shinpock, 1968); thymic extracts and thymocytes syngeneic with the recipient did not have this effect (Goodman & Shinpock, 1972). The enhancement was evident in erythroid, granuloid and thromboid tissues in the colonies (Goodman & Grubbs, 1970). In a parallel, but completely syngeneic, system thymocyte injection was shown to influence the growth of spleen colonies under certain circumstances (Lord & Schofield, 1973). Thus, addition of thymocytes to normal bone marrow or spleen suspensions did not enhance colony formation in sublethally-irradiated recipients but did enhance the colony-forming ability of irradiated marrow and spleen suspensions in these animals. These workers have therefore suggested that the co-operation of thymus cells is necessary for the growth of at least some of the colony-forming elements of bone marrow. Hrsak (1973) has also concluded that thymocytes have a trophic effect upon the growth of spleen colonies, despite the fact that bone marrow cellularity was unaffected by thymectomy in young adult life.

The proliferative characteristics of spleen colonies appear to closely resemble those of haemopoietic tissue <u>in situ</u> (Introduction, Sections 2.9 and 2.10) and thus it is clear that the effects of the thymus upon spleen colony growth which have been reported above might reflect a role for the organ during normal haemopoiesis. Thus it seemed pertinent to examine thymocyte proliferative activity during periods of enhanced bone marrow mitosis. Exogenous EPO (Chapter 2),

colbaltous chloride (Chapter 4), haemorrhage (Chapter 3) and haemolysed serum (Chapter 4) are erythropoietic stimulants which have been observed to produce detectable changes in bone marrow mitotic index and the rate of entry of bone marrow cells into mitosis. Thus these agents, with ADH and DDAVP which also increase bone marrow mitosis (Chapters 3 and 5), were suitable for the study of thymocyte proliferation during periods of enhanced bone marrow mitotic activity. *In vitro* culture of thymocytes with various hormones was also necessary to determine whether they might be directly mitogenic.

The experiments reported in this chapter were therefore designed to:

(i) Investigate the proliferation of thymocytes *in vivo* after the application of erythropoietic stimuli or agents which were known to stimulate bone marrow mitosis; and

(ii) Determine whether substances which enhanced thymocyte mitotic activity acted directly upon thymic elements or via a mediatory system.

Many of these studies were performed concomitantly with similar investigations upon bone marrow cellular proliferation which have been reported previously (Chapters 2, 3 and 4).

Methods.

Mitotic activity in rat thymic lymphocytes was measured in vivo at various times after the application of erythropoietic stimuli or other agents which were known to stimulate bone marrow cellular proliferation. Concomitant measurements of plasma calcium concentrations were also made in most experiments. The effects upon thymic lymphoblast proliferation of some hormones which were mitogenic in vivo were also examined in suspensions of thymocytes maintained in vitro.

Both the mitotic index and the rate of entry of cells into metaphase in the presence of Colcemid were assessed in the thymus (General Methodology, Section 6). Plasma total and ionised calcium concentrations were measured as before (Methodology, Sections 5.2 and 5.3).

A number of erythropoietic stimuli were applied to intact, and sometimes to hormonally-deprived, animals. EPO (Connaught Labs.) was dissolved in 0.9% saline and given as a single 0.5ml injection (10u/100g body weight) s.c. in the dorso-thoracic region. Albumin and α-globulin were administered as before (Methods, Chapter 2) to determine whether injection of plasma proteins could stimulate mitosis in a non-specific way. Cobaltous chloride (1 or 2.5 μmole/100g), an agent which stimulates endogenous EPO release (Jacobson et al., 1957b; Jacobson & Goldwasser, 1958), was administered s.c. as a single 0.5ml injection in a saline vehicle and the % of nucleated thymus cells which entered mitosis and reached metaphase over a 6h period was determined using Colcemid 1, 2 and 3 days later. Haemolysed serum, which has been claimed to stimulate endogenous EPO release (Erslev, 1971a), was prepared and administered as before (Methods Chapter 4) to normal and polycythemic rats. Mitotic index was measured 48 and 72h after

bleeding (2.0 - 2.5ml/100g by cardiac puncture), which is a stimulus for EPO release (Jacobson & Goldwasser, 1958; Fried <u>et al.</u>, 1970).

To determine whether the mitogenic action of ADH upon bone marrow cells 4h after haemorrhage (Chapter 3) was also evident in thymocytes, mitotic index and/or nucleated cell metaphase accumulation after Colcemid were determined in the thymus 4h after bleeding in normal, adrenalectomised (ADX), sham parathyroidectomised (sham PTX), parathyroidectomised (PTX) and hypophysectomised (HX) rats. Endocrine glands were excised, and animals maintained, as described previously (PTX, sham PTX — Methodology, Section 4.2; ADX — Chapter 3, Methods Supplement 3; HX — performed by commercial suppliers, Carworth Europe; see Chapter 3).

To confirm that thymus mitotic activity was stimulated by an ADH-mediated mechanism operating over the first 4h post-haemorrhage, rats were injected with ADH, (1-deamino-8-D-arginine)-vasopressin (DDAVP), aldosterone or angiotensin II amide with subsequent determination of mitotic index and/or the rate of accumulation of nucleated cells at Colcemid metaphase. The doses, routes of administration and suppliers of these agents have been stated earlier (Methods, Chapter 3).

To determine whether EPO or ADH in physiological concentrations could stimulate mitotic activity in thymocytes directly these hormones were added to suspensions of thymic lymphocytes maintained <u>in vitro</u> (see Methodology, Section 7.2 for description of technique). In some cases, thymocytes were cultured in Medium 199 (Wellcome Reagents Ltd.) and not B.G.S. medium. Smears were prepared and stained from the suspensions and scored for the % of nucleated cells in metaphase at 4 or 6h in the presence of 0.062mM Colcemid.

Table 41 overleaf.

Treatment	Thymus Mitotic Activity	
	Mitotic index	% Nucleated cells in metaphase at 6h
Saline	0.8 ± 0.2 (10)	6.2 ± 0.4 (5)
EPO (10u/100g)	1.4 ± 0.1 (5)	9.6 ± 0.5 (5)
Albumin (15.5mg/100g)	0.7 ± 0.1 (9)	-
α-Globulin (15.5mg/100g)	0.5 ± 0.2 (10)	-

Table 41: Effect of erythropoietin (EPO), albumin and α-globulin upon thymus mitotic activity at 24h. EPO, albumin and α-globulin were dissolved in 0.9% saline and injected s.c. in the dorso-thoracic region. 24h later, EPO had significantly elevated thymus mitotic index ($P < 0.001$) and the percentage of nucleated thymus cells which became arrested in metaphase in 6h in Colcemid (0.2mg/100g at 0 and 3h) - treated animals ($P < 0.001$). Albumin and α-globulin did not significantly affect thymus mitotic index at this time ($P > 0.5$ and $P > 0.2$ respectively). Numbers of animals indicated in parentheses.

Table 42 overleaf.

Treatment	% Nucleated Cells in Metaphase 6h after Colcemid		
	Day 1	Day 2	Day 3
Saline	6.3 ± 0.5 (3)	6.3 ± 0.4 (3)	6.6 ± 0.4 (3)
1 μmole/100g Co^{++}	7.1 ± 0.4 (5)	6.1 ± 0.4 (5)	8.9 ± 0.4 (5)
2.5 μmole/100g Co^{++}	7.2 ± 0.5 (5)	6.2 ± 0.3 (5)	8.6 ± 0.5 (5)

Table 42: Effect of cobalt (Co^{++}) upon thymus mitotic activity in rats. Cobaltous chloride ($CoCl_2$) was injected s.c. in a 0.9% saline vehicle in doses of 1 or 2.5 μmole/100g body weight. The percentage of nucleated thymus cells which entered mitosis and became arrested at metaphase in 6h after Colcemid (2 x 0.2mg/100g, at 0 and 3h) was determined 1, 2 and 3 days later. Both 1 and 2.5 μmole/100g Co significantly ($P < 0.001$ and $P < 0.01$ respectively) stimulated mitotic activity in the thymus on Day 3. Numbers of animals given in parentheses.

Table 43 overleaf.

Days after bleeding or sham-bleeding	Thymus Mitotic Index	
	Sham-bled	Bled
2	0.9 ± 0.1 (5)	1.5 ± 0.1 (6)
3	0.8 ± 0.2 (5)	1.45± 0.1 (5)

Table 43: Effect of haemorrhage upon thymus mitotic index at 2 and 3 days in rats. Animals were bled or sham-bled on Day 0. Thymus mitotic index was significantly higher in bled animals than in sham-bled controls on Days 2 and 3 ($P < 0.01$ in both cases). Numbers of animals indicated in parentheses.

Table 44 overleaf.

Treatment	Thymus Mitotic Index
Normal + serum	1.15 ± 0.1 (4)
Normal + haemolysed serum	1.45 ± 0.2 (5)
Polycythemic + serum	0.9 ± 0.1 (5)
Polycythemic + haemolysed serum	0.95 ± 0.2 (5)

Table 44: Effect of haemolysed serum upon thymus mitotic index in normal and polycythemic rats. Haemolysed serum (see Methods Chapter 4 for preparation) was injected i.p. on days 1 and 2 (2ml/100g body weight). Mitotic index was determined on day 4. Haemolysed serum did not significantly affect thymus mitotic index in either normal or polycythemic animals ($P > 0.1$ and $P > 0.5$ respectively). Numbers of animals indicated in parentheses.

Results.

Injection of exogenous EPO significantly increased mitotic activity in the thymus (Table 41) 24h later, in parallel with its effects upon bone marrow mitosis and plasma calcium concentrations (Table 24, Chapter 2). Injection of albumin and α-globulin, which are erythropoietically inactive (Labardini et al., 1968; see also Chapter 2), did not elevate thymus mitotic index (Table 41) over the same period.

Cobaltous chloride (1 or 2.5 μmoles/100g) significantly ($P < 0.001$ and $P < 0.01$ respectively) elevated the rate of accumulation of thymus cells at metaphase on the 3rd day after administration, but not on days 1 and 2 (Table 42). Again, this response parallels that of the bone marrow to the cobaltous ion (Table 34, Chapter 4). Similarly, the increase in mitotic index in the thymus observed 2 and 3 days after haemorrhage (Table 43) accompanied increases in bone marrow mitosis (Figure 37, Chapter 3) and also in plasma calcium concentration (Figure 38, Chapter 3). Thus exogenous EPO and two stimulants of endogenous EPO release (cobalt and haemorrhage) stimulated mitotic activity in the thymus in parallel with their effects upon bone marrow cellular proliferation and, in the cases of exogenous EPO and haemorrhage, plasma calcium concentration. However, the injection of haemolysed serum, which has been claimed to stimulate the release of endogenous EPO (Erslev, 1971a, did not increase mitotic index in the thymus (Table 44) in either normal or polycythemic rats, although it did elevate marrow mitotic index (Figure 50, Chapter 4). This must be taken to indicate that haemolysed serum does not truly stimulate EPO release under the conditions pertaining to the studies (see Discussion, Chapter 4).

Four hours after haemorrhage there is a significant increase in the mitotic activity of bone marrow which is independent of the release

Treatment	Thymus Mitotic Index
Normal + sham-bleeding	0.9 ± 0.1 (4)
Normal + bleeding	1.4 ± 0.1 (4)
Sham PTX + sham-bleeding	0.8 ± 0.1 (5)
Sham PTX + bleeding	1.35± 0.2 (5)
PTX + sham-bleeding	0.4 ± 0.1 (5)
PTX + bleeding	0.9 ± 0.1 (5)

Data plotted as Figure 54, overleaf. Numbers of animals given in parentheses.

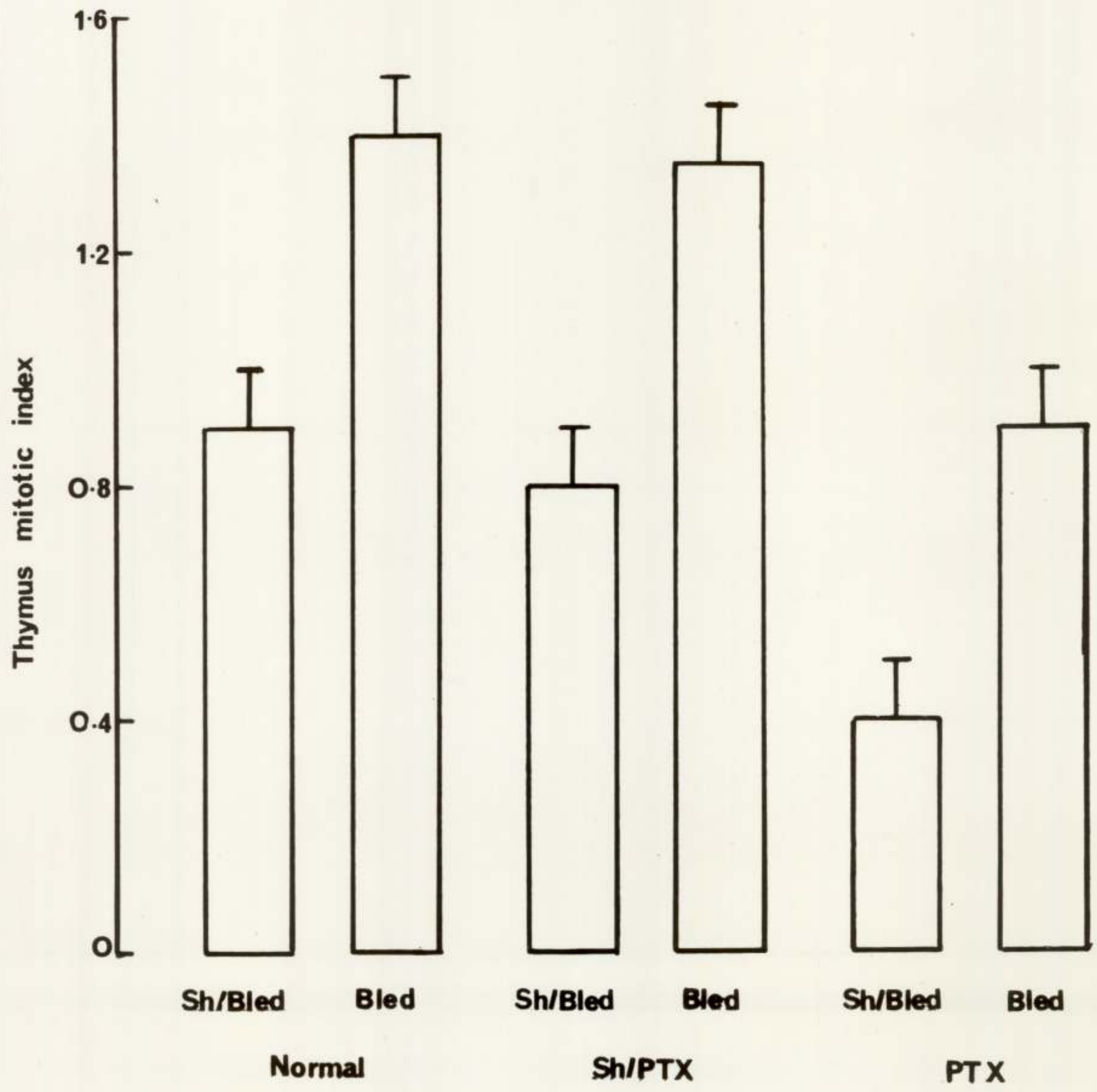


Figure 54: Effect of haemorrhage upon thymus mitotic index at 4h in normal, sham-parathyroidectomised (Sh/PTX) and parathyroidectomised (PTX) rats. Animals were bled (2.0 - 2.5ml/100g body weight) or sham-bled (Sh/Bled) at 0h. Thymus mitotic index was significantly elevated at 4h in normal ($P < 0.01$), sham-PTX ($P < 0.05$) and PTX ($P < 0.01$) animals. Columns and vertical bars are means + s.e.m. from 4-5 animals in each case.

Table 45 overleaf.

Treatment	Thymus Mitotic Index
ADX + sham-bleeding	0.7 ± 0.1 (5)
ADX + bleeding	1.05± 0.1 (7)

Table 45: Effect of haemorrhage upon thymus mitotic index in adrenalectomised (ADX) rats at 4h. ADX rats were bled (2.0 - 2.5ml/100g body weight) or sham-bled at OH. 4h later the mitotic index in the thymus was significantly ($P < 0.01$) greater than that in sham-bled controls. Numbers of animals given in parentheses.

Table 46 overleaf.

Treatment	Thymus Mitotic Activity at 4h	
	Mitotic Index	% Nucleated Cells in Metaphase
Normal + sham-bleeding	0.4 ± 0.1 (4)	4.8 ± 0.2 (4)
Normal + bleeding	0.75± 0.1 (5)	6.1 ± 0.3 (5)
HX + sham-bleeding	0.45± 0.2 (5)	3.9 ± 0.3 (5)
HX + bleeding	0.3 ± 0.2 (7)	2.6 ± 0.4 (6)

Table 46: Effect of haemorrhage upon thymus mitotic activity in normal and hypophysectomised (HX) rats at 4h. Animals were bled (2.0-2.5ml/100g body weight) or sham-bled at 0h. Haemorrhage significantly increased thymus mitotic index and the percentage of nucleated cells accumulated at metaphase (after 0.22mg /100g Colcemid) at 4h in normal rats ($P < 0.05$ and $P < 0.01$ respectively) but not in HX rats. The rate of accumulation of thymocytes at Colcemid metaphase significantly decreased in HX rats ($P < 0.05$), though mitotic index did not ($P > 0.2$). Numbers of animals given in parentheses.

Treatment	Thymus Mitotic Index
Saline	0.6 ± 0.1 (14)
ADH	1.1 ± 0.1 (6)
DDAVP (75pg/100g)	1.1 ± 0.2 (5)
Aldosterone	0.6 ± 0.3 (5)
Angiotensin II	0.6 ± 0.2 (5)

Data plotted as Figure 55, overleaf. Numbers of animals given in parentheses.

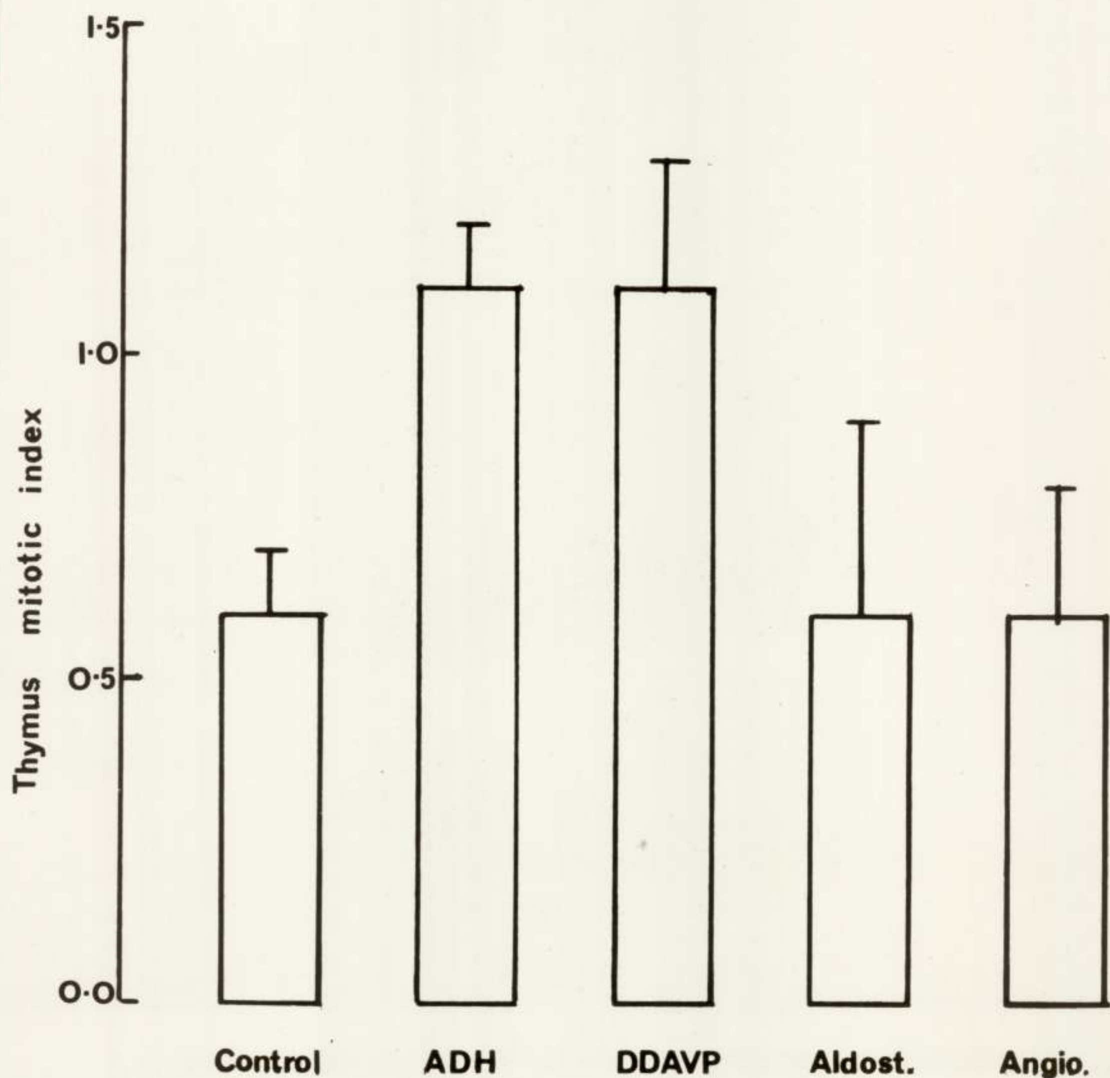


Figure 55: Effect of hormones upon thymic mitotic index at 4h in rats. Anti-diuretic hormone (ADH) and (1-deamino-8-D-arginine)-vasopressin (DDAVP) significantly increased thymus mitotic index ($P < 0.01$ and $P < 0.05$ respectively) but aldosterone and angiotensin II amide did not. Doses and routes of administration are as described in Methods, Chapter 3. Columns and vertical bars represent means + s.e.m. from 5-14 animals in each case.

of EPO and the extracellular status of the calcium ion (Chapter 3). This pattern is also observed in the thymus, for the mitotic index of the tissue is elevated after haemorrhage in normal, sham PTX and PTX rats (Figure 54). The increase in bone marrow cellular proliferation has been ascribed to the action of ADH, which is rapidly released in massive amounts after haemorrhage (Chapter 3). To ascertain whether this hormone is also responsible for the mitotic stimulation observed in the thymus, a similar pattern of experiments to those which have been reported in Chapter 3 were performed. ADH and DDAVP both significantly elevated mitotic index (Figure 55) and the rate of accumulation of nucleated cells at Colcemid metaphase (Figure 56) within 4h. Aldosterone and angiotensin II did not elevate thymic mitotic index over the same period (Figure 55). Adrenalectomy did not reduce the mitotic stimulation observed in the thymus at 4h (Table 45), suggesting that adrenal hormones were not mitogenic in this situation. However, hypophysectomy completely abolished any increment in either mitotic index or the percentage of nucleated cells reaching metaphase at 4h in the thymus (Table 46). Indeed, the latter indicator of mitotic activity actually decreased significantly ($P < 0.05$) over this period, in parallel with plasma calcium concentration (Figure 39, Chapter 3). All these observations paralleled those made in bone marrow (Chapter 3) and strongly suggest that ADH is responsible for these changes.

To determine whether ADH, EPO, aldosterone and angiotensin II could directly stimulate thymocyte proliferation at concentrations similar to those ambient after haemorrhage (see Chapter 3), the hormones were added to suspensions of thymic lymphocytes maintained <u>in vitro.</u> EPO, aldosterone and angiotensin II had no effects upon the entry of

Treatment	% Nucleated thymus cells in metaphase 4h after Colcemid
Control	4.2 ± 0.2 (8)
ADH (6mU/100g)	5.7 ± 0.4 (5)
DDAVP (75pg/100g)	6.0 ± 0.2 (5)
DDAVP (100pg/100g)	6.2 ± 0.2 (5)

Data plotted as Figure 56, overleaf. Numbers of animals indicated in parentheses.

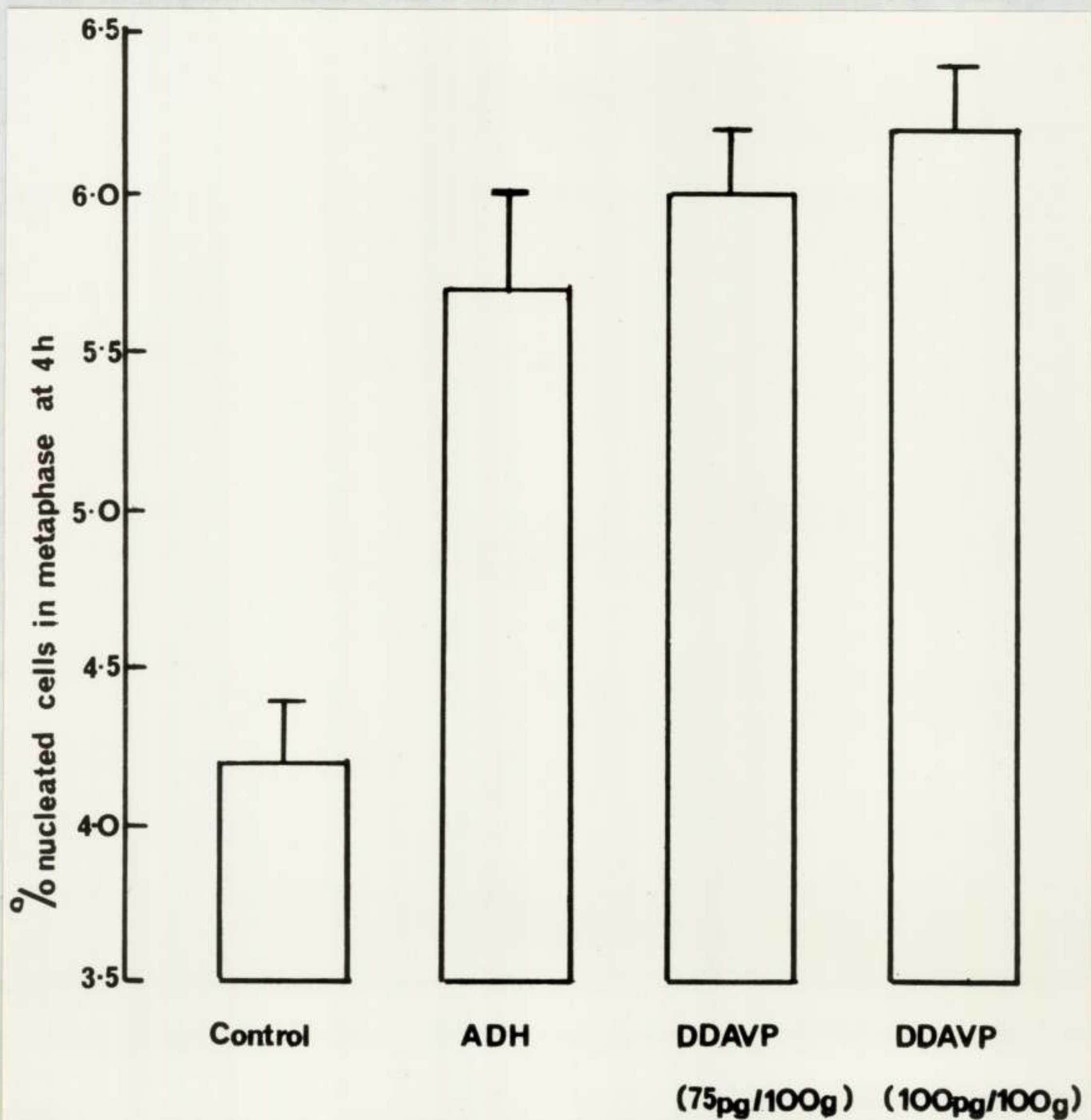


Figure 56: Effects of anti-diuretic hormone (ADH) and (1-deamino-8-D-arginine)-vasopressin (DDAVP) upon mitotic activity in the thymus of normal rats at 4h. ADH and DDAVP were administered intracardially in 0.1ml 0.9% saline at 0h. Controls received either saline or saline + mannitol (see Figure 45); values were pooled since mannitol did not affect thymus cellular proliferation. ADH and both doses of DDAVP significantly ($P < 0.01$ in all cases) elevated the percentage of nucleated thymus cells which reached the arrested metaphase condition 4h after Colcemid (0.22mg/100g body weight). Columns and vertical bars represent mean + s.e.m. from 5-8 animals in each case.

Table 47 overleaf.

Treatment	% Nucleated thymus cells in metaphase	
	4h	6h
Untreated	3.3 ± 0.2	4.3 ± 0.3
ADH (0.6mU/ml)	5.0 ± 0.3	7.0 ± 0.3
ADH (0.6mU/ml) + Imidazole (0.5mM)	3.6 ± 0.4	4.4 ± 0.4
ADM (0.6mU/ml) + Caffeine (0.4mM)	6.0 ± 0.3	8.2 ± 0.3
EPO (0.25U/ml)	3.6 ± 0.4	4.4 ± 0.4
EPO (0.5U/ml)	3.1 ± 0.3	3.9 ± 0.5
Angiotensin II (3ng/ml)	3.0 ± 0.4	4.0 ± 0.3
Aldosterone (5ng/ml)	3.5 ± 0.2	4.3 ± 0.4

Table 47: Effects of hormones upon thymocyte proliferation in vitro at 4 and 6h. Erythropoietin (EPO) at two different concentrations, angiotensin II and aldosterone failed to increase the flow of nucleated cells into metaphase (in the presence of 0.062mM Colcemid) at 4 or 6h. Anti-diuretic hormone (ADH) significantly increased the rate of accumulation of nucleated cells in metaphase at 4h ($P < 0.01$) and 6h ($P < 0.001$). This action was abolished in the presence of imidazole ($P > 0.5$ over both periods) but potentiated by caffeine (increment significant at 6h - $P < 0.05$). Values are means ± s.e.m. from 4 cultures in each case.

thymocytes into metaphase in the presence of Colcemid (Table 47). However, ADH significantly ($P < 0.01$) increased thymic lymphoblast cellular proliferation. Furthermore, this action was inhibited by 0.5mM imidazole and potentiated by 0.4mM caffeine (see Chapter 5 for detailed discussion of these two agents) which strongly suggests that the effect was mediated via intracellular cyclic adenosine 3', 5'-monophosphate (cyclic AMP).

Discussion.

The scattered observations which have linked the thymus with the formation of blood cells (see Introduction, this Chapter) suggested that changes in thymopoiesis might follow erythropoietic challenge. These changes have been observed (Tables 41 - 46, Figures 54 - 56) but it is not clear whether they are truly related to the enhanced proliferation of the bone marrow cells or are consequences of non-specific stimulation by the hormonal and ionic (i.e. EPO and calcium) mediators of the erythropoietic stimuli.

Exogenous EPO and the endogenous EPO which was presumably released in response to cobalt (Jacobson & Goldwasser, 1958; Rodgers et al., 1972) and haemorrhage (Fried et al., 1970) produced increases in thymocyte mitotic activity, although these were delayed in the case of cobalt and haemorrhage (see Chapters 4 and 3, respectively, for discussion). However, since equivalent concentrations of EPO are incapable of directly stimulating thymocyte mitosis in vitro it seems likely that these agents exert their effects via the parathyroid-dependent elevations of mobile calcium which have previously been demonstrated to follow their application (Chapters 2,3 and 4). This conclusion is further substantiated by the observation that haemolysed serum, which stimulates bone marrow proliferation but does not increase plasma calcium concentration (Chapter 4), does not increase thymus mitotic index. Further investigations of this important observation are needed, for it suggests two conclusions:

(i) In three situations where elevated circulating EPO levels are apparent, increased mitotic activity is observed in the thymus (Tables 41 - 43). However, no such elevation is observed after the administration of haemolysed serum, which would tend to substantiate the hypothesis (Chapter 4) that the increases in circulating EPO reported by

Erslev after administration of haemolysed serum were in some way artefactual.

(ii) Haemolysed serum does stimulate erythropoiesis in the bone marrow (Labardini et al., 1968; Chapter 4), and thus in this situation there is increased erythropoietic activity without concomitant elevation of thymocyte mitotic activity. This infers that the parallel increases in bone marrow and thymocyte proliferation which have been observed (Tables 41 - 43) are functionally distinct, i.e. that thymocyte mitosis is stimulated in a non-specific way by elevations of plasma calcium concentrations. This conclusion is supported by the well established mitogenic actions of the calcium ion in physiological ranges in vitro (Whitfield & Youdale, 1966; Whitfield et al., 1969b; MacManus & Whitfield, 1971), where there is of course no influence from erythropoietic tissue.

The observations that ADH and its specific analogue DDAVP were both capable of stimulating thymocyte mitosis within 4h (Figures 55 and 56) suggested that a mechanism might operate on the thymus after haemorrhage which was similar to that which has been shown to stimulate bone marrow cellular proliferation in the same situation (Chapter 3). This was confirmed by a series of experiments identical to those which have been reported in Chapter 3. Thus, injection of aldosterone and angiotensin had no effect upon thymopoietic divisions (Figure 55). Adrenalectomy did not affect the 4h response of the thymus to haemorrhage (Table 45) whereas HX completely abolished this response (Table 46). Taken in toto, these results suggest that increased circulating levels of ADH stimulate thymocyte proliferation within 4h of haemorrhage.

ADH probably stimulated thymocyte proliferation in vivo by a direct action for it was capable of this effect in vitro in concentrations equivalent to those present in the plasma after haemorrhage (Table 47).

EPO, aldosterone and angiotensin II were not, however, effective *in vitro* (Table 47). The mechanism of action of ADH appeared to be mediated via cyclic AMP, for imidazole severely reduced the effect of ADH while caffeine significantly ($P < 0.05$) potentiated it (see Chapter 5 for discussion). It should be noted that this effective dose (0.6mU/ml) of ADH, which was equivalent to the endogenous circulating levels observed immediately after haemorrhage (see Chapter 3), is only 1/70 of the concentration which has previously been demonstrated to be mitogenic in thymocyte cultures (Whitfield *et al.*, 1969a).

It seems, therefore, that the increases in thymocyte proliferation which have been observed concomitantly with increased bone marrow mitosis are produced by the mitogenic influences of elevated extracellular calcium ion or ADH concentrations rather than by a bone marrow-dependent factor. Thus the parallel changes in bone marrow and thymus mitotic activity which have been observed in a variety of situations in which the ambient ionised calcium concentration varies (e.g. circadian rhythms, Chapter 1; also $CaCl_2$ or PTH injection and PTX, Introduction Section 5 and Table 1) probably do not depend upon the increased migration of stem cells from bone marrow to thymus but rather reflect a direct action of the ion (Table 1; also Chapter 5). However, this does not preclude the possibility that elevations in thymocyte cell division *in vivo* do influence bone marrow mitosis, for the calcium homeostatic system is a true control system for erythropoiesis (Chapters 1-4; also Table 1). The demonstration of a situation in which bone marrow mitosis can be elevated without accompanying changes in thymocyte proliferation (Table 44 and Figure 50, Chapter 4) perhaps lessens the possibility of a role of the thymus in haemopoiesis. This can only adequately be ascertained in thymectomised animals, though any changes in erythroid capacity might be too subtle to be detectable by the methods which have been used in this present study.

Summary.

Exogenous EPO, cobaltous chloride and haemorrhage have been demonstrated to stimulate thymocyte division in parallel with their effects upon bone marrow. Haemolysed serum injection did not influence thymopoiesis.

Both endogenous and exogenous ADH elevated mitotic activity in the thymus within 4h. The effect of haemorrhage at 4h upon thymocyte proliferation was similar to that upon bone marrow mitosis.

EPO, aldosterone and angiotensin, in physiological concentrations, did not influence the rate of cell division in thymocyte suspensions maintained *in vitro*. ADH was mitogenic in this system; the mechanism of action of the hormone was dependent upon mediation by cyclic AMP.

It was concluded that enhanced rates of mitosis in bone marrow and thymus occurred independently in the situations which have been examined. The possibility of a thymic influence upon haemopoiesis has not been substantiated but cannot be disregarded.

DISSERTATION

The role of hormones and ions in the physiological control of cellular proliferation in rat erythropoietic and lymphopoietic tissues.

"And if my thought streams could be seen
They'd probably put my head in a guillotine...
It's alright, Ma, it's life and life only...."

Bob Dylan, It's Alright Ma
(I'm Only Bleeding)

The observations which have been reported in this study have been thoroughly discussed as they have been presented and will therefore not be considered specifically in this dissertation. It is, however, necessary to attempt to evaluate their overall significance for the animal and to suggest further work which is necessary to confirm their physiological pertinence.

The major findings fall into three categories:

(i) Those which demonstrate further parallels between the extracellular calcium concentration and the level of mitotic activity in bone marrow and/or thymus, i.e. during circadian rhythms and after haemorrhage or the administration of exogenous EPO, cobalt or calcitonin. It should be emphasized that several of these phenomena depend upon the hypercalcaemic actions of EPO, either endogenous or exogenous.

(ii) Mitogenic agents which act directly upon cells in the two tissues, i.e. ADH and probably haemolysed serum.

(iii) Mechanistic studies upon the mitogenicity of ADH and the calcium ion *in vitro*.

The conclusions which can be drawn from these experiments are manifold, but the most significant may be summarised:

(i) The calcium homeostatic system, via modulations in the extracellular calcium concentration, is a true control system for erythropoietic and lymphopoietic tissues.

(ii) The hormone EPO possesses, in addition to its direct actions upon marrow erythroid elements, the capacity to elevate the level of calcium in the plasma. It seems that this latter property is essential for the manifestation of EPO's mitogenic properties after strong erythropoietic stimuli.

(iii) A link between the endocrinological responses to loss of blood volume and to loss of erythrocytes has been established by the demonstration of a mitogenic action of the anti-diuretic hormone after haemorrhage.

(iv) Proliferation in the thymus has been shown to parallel that in the bone marrow under most circumstances.

(v) Doubt has been cast upon the assertion that haemolysed serum may stimulate release of EPO.

It is particularly striking to note that the changes in plasma calcium concentration which are associated with variations in bone marrow or thymus proliferation are in many cases small. Often increases of less than 10% are capable of elevating marrow mitotic activity by 50% or more. This of course emphasizes the importance of the very precise control exerted by the calcium homeostatic system upon the free calcium in the body. Certainly, the rate of cell division in the test tissues appears to be very sensitive over a range extending 10% on either side of the plasma homeostatic norm; the necessity for large changes in mobile calcium to effect regulation of cellular proliferation would be distinctly physiologically disadvantageous for many other processes are critically dependent upon the regulation of calcium in the body fluids.

To extend the calcium/proliferation relationship which exists over the critical range down to the plasma calcium levels which are extant after parathyroid gland removal would predict that mitosis would cease altogether after the excision. This does not happen and in practice it is difficult to depress bone marrow proliferation by more than 50-60%. This suggests either that there is a residue of cells which are not sensitive to activity of the calcium ion, that the

extracellular calcium concentration is only critical over the range near normal levels or that compensatory mechanisms exist. The observation that CT administration can further depress mitosis in aparathyroid animals would normally be evidence that the first suggestion above was invalid; however, there were several unusual features inherent in the experiment (Chapter 4). In the case of the bone marrow it is clear that EPO, possibly in conjunction with factors inherent in the bone marrow environment (i.e. reticular elements), still operates in the absence of the parathyroid gland; for example, haematocrit is re-established in parathyroprivic rats after haemorrhage (Perris _et al_. 1971). It is just as clear that the presence of the parathyroid is necessary for the rapid stimulation of erythropoiesis by EPO (c.f. Perris _et al._, 1971; Chapters 2 and 4).

It should be acknowledged here that the assumption is continually made that the _extracellular_ calcium concentration is directly related to the _plasma_ calcium concentration. This is perhaps best justified on empirical grounds, i.e. changes in plasma calcium status have been repeatedly, and unfailingly, shown to accompany changes in cellular proliferation in the bone marrow and thymus. Certainly there is no reason to suspect that the extracellular microenvironment does not reflect changes in plasma calcium. However, the extracellular fluid probably contains less protein and less total calcium than the plasma (c.f. Rasmussen, 1970) and thus equivalent changes in total calcium concentration will produce larger shifts in ionised calcium concentration in the extracellular fluid than in the plasma (specimen calculations have shown the validity of this statement). This could of course explain why, as mentioned above, small shifts in plasma calcium

concentration produce large changes in cellular proliferation. The environment of cells is critical for the expression of their capabilities and it is natural to speculate whether the bone marrow could be an unusual tissue in its response to calcium fluctuations by virtue of its location within bone (i.e. within the tissues in which constant calcium exchange with plasma is proceeding). Certainly the proliferation of bone marrow is drastically reduced _in vitro_ but this is a common phenomenon in tissue culture. Since the thymus, and latterly the liver, have been shown to also be influenced by extracellular calcium status it is unlikely that the bone marrow is unique because of its anatomical location.

The concept of the calcium homeostatic system exerting control over tissue proliferation raises several problems. For example, does the elevation of plasma calcium by EPO also stimulate mitosis in all calcium-susceptible tissues? This parallelism is observed in the thymus (Chapter 6) but has not been studied in gut mucosa. The thymus is a somewhat special case, in that it is seeded by bone marrow precursors and could possibly have some reciprocal influence upon haemopoiesis. However the gut mucosal epithelium and the skin (a rapidly proliferating tissue which has not, as yet, been examined for a response to calcium elevations) are tissues in which enhanced proliferation after erythropoietic stimuli would be difficult to justify physiologically. There is insufficient evidence available to speculate upon this point at present.

The control exerted by calcium homeostasis upon erythropoietic proliferation is more easily understood. EPO is envisaged as acting directly upon erythroid elements and also as elevating the extracellular calcium concentration. Calcium-induced mitogenesis could occur at

pluripotential stem cell level or within the erythroid series to provide a larger base of cells upon which EPO might exert its differentiation and maturation effects. This system also explains how a feedback system may act upon calcium homeostasis in this situation: when the oxygen supply/demand ratio is re-established EPO production will cease and plasma calcium concentration return to normal levels. This still leaves the problem of how plasma calcium concentrations are maintained at supranormal levels for long periods, for although EPO produces and sustains the elevation it is difficult to see why increased CT release and decreased PTH release do not act to return plasma calcium concentrations to normal levels.

The demonstrations that calcium homeostasis may also be involved in the regulation of intestinal epithelial proliferation (Mellon, 1974) and liver regeneration (Rixon & Whitfield, 1972b) bear out the parallels which were established between plasma ionised calcium concentration and rapid body growth in young animals (Perris et al., 1968). It is not clear at the present time whether calcium may stimulate mitosis in all cells which are capable of division or whether this action is limited to rapidly-proliferating and regenerating tissues. The granuloid series within the bone marrow certainly appears to be a cell population which is not dependent upon calcium ion status (Rixon & Whitfield, 1972a). It is quite feasible that non-dividing tissues, or those which are not susceptible to the mitogenic actions of calcium, do not possess one of the intracellular factors which have been postulated to be necessary for the expression of calcium's action (Figure 4). Of course it is possible that all cells which are capable of division are susceptible to calcium but that they have differing membrane permeabilities to the ion. Calcium has been postulated to enter

the cell, rather than to act upon an external receptor, to exert its mitogenic action because some agents which increase membrane stability inhibit calcium-induced proliferation in vitro (Whitfield et al., 1969a).

Under normal, in vivo, conditions the extracellular calcium environment does not change substantially. Thus Whitfield (Whitfield et al., 1973c) has postulated that the cell itself may initiate changes in membrane permeability to the ion at appropriate stages of the cell cycle, i.e. that calcium effects all in vivo mitotic events (in thymocytes at least) without the necessity for changes in the extra-cellular calcium concentration. The mitotic activity observed in thymocytes maintained in calcium-free medium is ascribed (Whitfield et al., 1973c) to cells in which DNA synthesis has been initiated prior to isolation. An alternative explanation, of course, is that not all mitotic events are stimulated by the calcium ion. It would be interesting to study the pattern of mitosis with time in calcium-free medium. Since elevated calcium concentrations (greater than 1.0mM) produce a significant increase in thymocyte proliferation in vitro within 4h it would be expected that the progression of cells into Colcemid metaphase would cease after 4-5 hours in calcium-free medium. Examination of the data of Whitfield et al. (1969a) reveals several situations in which there is a reduction in the rate of accumulation of cells at metaphase between 4 and 6h and one where there is a complete cessation from 5-7h. Similar results may be observed in the work of MacManus et al. (1971a). However, the demonstration is not clear cut and this crucial point deserves further investigation.

If the calcium ion does initiate all tissue mitoses it is possible to envisage a well-regulated system in which the basic, endogenously regulated mitotic activity is influenced by factors emanating from

outside the individual tissue (e.g. elevated calcium levels, EPO). Contact inhibition might be a prime determinant of the endogenous regulation and it is also not possible to rule out the chalone theories. If chalones (tissue specific mitotic inhibitors - Bullough, 1962) do exist it is not impossible that they could act by restricting calcium entry to the cell. If they are tissue specific it is likely that such an action would be at the source (probably nuclear) of the endogenous stimulus which cues increased calcium entry to the cell rather than upon cell membranes.

The mechanism of action which has been described for calcium upon thymic lymphoblast proliferation (General Introduction, Section 5.4; Figure 4) has some parallels in bone marrow cells (Chapter 5). Certainly the involvement of intracellular cyclic AMP in the mediation of the actions of calcium and hormones upon bone marrow mitosis warrants further investigation. However, this involvement of cyclic AMP cannot yet be said to be fully explained. The main stumbling-block is the ubiquitous nature of cyclic AMP itself. A great many hormone-cell interactions have been reported to be mediated via the "second messenger" cyclic AMP (c.f. Robinson et al., 1971). A very basic point which has not been explained is this: if a target cell is capable of producing responses to the actions of two separate hormones, both of which are mediated via cyclic AMP, then how does the cell "decide" which response is required? Of course, different hormones will act upon different membrane receptors which are structurally specific for individual hormones. However, the intracellular cyclic AMP elevation which is induced by a hormone must be distinguishable from that produced by others and it is simply not apparent how this is achieved. This is of particular importance when the mitogenic mechanism induced by calcium is considered. Clearly it is not desirable that mitogenic events are initiated by any hormonal stimulus which is mediated via cyclic AMP. It might perhaps be considered that

all highly differentiated cells which are concerned with the synthesis of particular proteins are incapable of mitogenic stimulation via cyclic AMP, but this seems very unlikely. Possible explanations are:

(i) Refinements may exist within the cyclic nucleotide structure, and similar, though distinct, nucleotides may be produced in response to different stimuli. In this context it is interesting to note that another cyclic nucleotide, cyclic guanosine 3', 5'-monophosphate, is now being implicated with the events governing mitogenic stimulus (Whitfield & MacManus, 1972; Morgan, personal communication).

(ii) Hormone receptors may have a distinct adenyl cyclase complex associated with them, with an activation site in close proximity. One hormone might locally elevate cyclic AMP formation which would trigger the release of imitiating substances from the associated activation site. This mechanism is probably unlikely, for localised cyclic AMP production of this nature would presumably be undetectable ; tissue cyclic AMP elevations have been demonstrated in many situations after the application of suitable stimuli (Robinson *et al.*, 1971).

It is quite possible that present assay techniques are too insensitive to distinguish between structurally similar nucleotides and that "cyclic AMP elevation" covers a multitude of events. This is certainly more feasible than the possibility that the mechanism (Whitfield *et al.*, 1973c) for the mitogenic action of calcium is merely an artefact of *in vitro* culture. This point has been discussed previously (Chapter 5).

The demonstration of a link between the response of the animal to loss of blood volume and to loss of red cells, i.e. the mitogenic action of ADH after haemorrhage, is interesting but does not reveal which bone marrow series is responsive. This weakness can be overcome by studying the radioiron incorporation of bone marrow cells *in vitro*

in the presence of ADH to determine whether the hormone stimulates erythropoiesis solely, non-specifically, or not at all. Radioiron incorporation has not been measured at any time in the present studies; however, it is clear that the mitotic response of the bone marrow to erythropoietic stimuli must reflect increased erythropoiesis.

The present studies have all been conducted in male rats. A number of reports (e.g. Smith et al., 1974) have suggested that proliferative control in female rats may differ in degree, though possibly not in mechanism, from that in males. Personal, unpublished observations showed one such instance: female rats did not show circadian variations in plasma calcium and tissue mitosis under the same conditions in which such changes were clear-cut in males (Chapter 1). Ovariectomy restored the male pattern of variation. It is clear that observations on the response of female rats to various erythropoietic stimuli, ADH, etc. would be of considerable physiological interest.

The clinical significance of these observations has not been investigated, but two approaches are self-evident:

(i) Measurement of plasma calcium concentrations in one of the many conditions of anaemia in which circulating EPO levels are supranormal.

(ii) Investigation of the blood cellularity of patients suffering from hypoparathyroidism or vitamin D deficiency diseases.

The significance of the mitogenic role of ADH after haemorrhage in the rat is less open to investigation in man, for, although diabetes insipidus is characterised by a failure to synthesize ADH, the long-term effects of haemorrhage in hypophysectomised rats have not yet been studied.

A number of lines of investigation suggested by the work reported here have been mentioned previously. Three other areas particularly demand study:

(i) It is necessary to establish the types of erythroid cell which are acted upon by the calcium ion. This could probably be inferred from two complementary investigations. The exogenous and endogenous colony-forming assays could be performed in conditions of varying extracellular calcium status; mitogenic actions of calcium would probably be reflected in the number of colonies formed after administration of consistent numbers of bone marrow cells. Conversely myeleran, which suppresses the activity of pluripotential stem cells (Reissmann & Samorapoompichit, 1970), could be used to study the effects of calcium upon erythroblasts.

(ii) It would be of value to study the changes in peripheral blood cells, both red and white, after haemorrhage in intact animals and in those which had no PTH or EPO activity. The former condition can be achieved by PTX and the latter by nephrectomy or administration of neuraminidase or anti-EPO immune serum. As mentioned previously (Chapter 3) these methods of suppressing EPO activity have drawbacks; however, parallel studies using all three should reduce the possibilities of artefactual results.

(iii) It would be interesting to study the effects of haemorrhage upon short-term bone marrow proliferation in Brattleborough rats which are congenitally incapable of producing ADH.

The studies which have been reported here further extend the concept that the calcium homeostatic system is closely bound up with the control of cellular proliferation in bone marrow and thymus. Further work is undoubtedly required to investigate the subtleties of such a mechanism, but the observations of the mode of action of EPO show that

the control exerted by the calcium homeostatic system can be precise and well-regulated. This further physiological role for the calcium homeostatic system must now be considered to be established.

My friends at the prison, they ask unto me
"How good, how good, does it feel to be free?"
And I answer them most mysteriously
"Of birds freed from the chains of the skyway..."

Bob Dylan, Ballad in Plain D.

APPENDIX I

Values of test parameters in normal, saline-injected or sham-bled (see Table 25) rats.

Normal values:

Parameter	Mean	s.e.m.	Range	No.
[1]Total calcium (mg/100ml)	10.1	0.04	8.5 - 11.7	246
[1]Ionised calcium (mg/100ml)	5.0	0.04	4.2 - 5.8	121
[1]Inorganic phosphate (mg/100ml)	7.8	0.17	5.3 - 9.8	52
[1]Total magnesium (mg/100ml)	2.0	0.05	1.1 - 2.3	31
[1]Protein (g/100ml)	8.2	0.11	6.5 - 9.9	35
[2]Haemoglobin (g/100ml)	13.3	0.14	11.1 - 14.2	16
Haematocrit	42	0.55	29 - 56	74
[3]Bone marrow	1.3	0.03	0.6 - 2.3	156
[3]Thymus	0.8	0.04	0.3 - 1.4	58
[4]Bone marrow	15.6	0.62	11.0 - 21.3	24
[4]Thymus	7.3	0.36	5.3 - 10.1	18
[5]Bone marrow	10.9	0.26	6.6 - 15.4	67
[5]Thymus	5.3	0.14	3.4 - 7.8	67

Notes: 1 = plasma concentration, 2 = blood concentration, 3 = mitotic index, 4 = nucleated cells in Colcemid metaphase at 6h, 5 = nucleated cells in Colcemid metaphase at 4h.

APPENDIX II

Compositions of normal and calcium-deficient diets and tap water.

Constituent	Average content (% by weight or stated)	
	Normal	Calcium Deficient
Ca	1.20	-
P	0.85	0.38
NaCl	0.82	0.73
Mg	0.30	0.52
K	0.83	0.38
Fe	0.018	0.04
Co^{++}	0.010	Trace?
Digestible Protein	15.7	(Total) 24%
Digestible Oils	2.38	(Total) 5%
Digestible Carbohydrate	45.9	(Total) 68%
Vitamins A, B_1, B_2, B_6, B_{12}, E, K, D_3 also supplied.		

Table 48: Abbreviated constitutions of diets. For full details Pilsbury's Ltd. and Kenny & Munson, 1959.

Chemical Results	Concentration in Tap Water (ppm)	
	Mean	Range
Ammoniacal Nitrogen	0.04	0 - 0.09
Albuminoid Nitrogen	0.12	0.03- 0.24
Nitrite Nitrogen	0.001	0 - 0.002
Nitrate Nitrogen	0.60	0 - 2.50
Free CO_2	1.50	0 - 4.50
$CaCO_3$	24.00	20.00- 31.00
Chlorides	9.00	7.00- 11.00
Phosphates (as Phosphorus)	0.05	0.01- 0.25
Iron	0.17	0 - 0.40
Potassium	0.42	0.15- 0.70
Sodium	3.73	1.15- 5.60
Fluorides	0.93	0.70- 1.05
Manganese	0.01	0 - 0.04
pH	7.8	7.1 - 8.6

Table 49: Composition of tap water. Source, City of Birmingham Water Department.

APPENDIX III

Suppliers of materials.

Bantin and Kingman Ltd., Goole, Yorks., U.K.

British Drug Houses Ltd., Poole, Dorset, U.K.

Carworth Europe, Alconbury, Hunts., U.K.

CIBA Chemicals, Horsham, Sussex, U.K.

Connaught Laboratories, Willowdale, Ontario, Canada.

Coulter Electronics, Hialeah, Florida, U.S.A.

Evans Electrosolenium Ltd., Halstead, Essex, U.K.

Fisons Ltd., Loughborough, Leics., U.K.

Orion Research Inc., Cambridge, Mass., U.S.A.

Parke-Davis, Co. Ltd., Pontypool, Glam., U.K.

Philip Harris Ltd., Birmingham, Warks., U.K.

Pilsbury's Ltd., Birmingham, Warks., U.K.

Reckitt and Colman Ltd., Hull, Yorks., U.K.

Schwartz-Mann Co., New York, N.Y., U.S.A.

Scientific Products Farm Ltd., Canterbury, Kent, U.K.

Sigma Chemicals, St. Louis, Mo. U.S.A.

Sterilin Products, Richmond, Surrey, U.K.

Wilson Laboratories, Chicago, Ill., U.S.A.

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ERYTHROPOIETIN-INDUCED CHANGES IN PLASMA CALCIUM AND BONE MARROW MITOSIS IN THE RAT

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SUMMARY

Subcutaneous injections of ovine erythropoietin preparations caused parallel increases in bone marrow mitotic activity and plasma calcium concentrations. These activities appeared to be a specific property of the erythropoietin molecule and were not due to an impurity. The increased mitosis and hypercalcaemia which followed erythropoitein injection in normal rats did not occur in the aparathyroid animal. Although a functional parathyroid gland was essential for the erythropoietin-induced changes, the mode of interaction between erythropoietin and the parathyroid gland remains to be established.

INTRODUCTION

The concentration of calcium in the extracellular environment determines the rate of progression into DNA synthesis of a wide variety of cells and their subsequent entry into mitosis. This is true not only of cells isolated and maintained *in vitro*, but also of bone marrow and thymus cells in the whole animal (Hollingsworth, 1941; Tyler, 1941; Heilbrun, 1952; St Amand, Anderson & Gaulden, 1960; Whitfield & Rixon, 1962; Whitfield & Youdale, 1966; Fautrez-Firlefyn & Fautrez, 1967; Perris & Whitfield, 1967; Perris, Whitfield & Rixon, 1967; Morton, 1968; Rixon, 1968; Whitfield, Rixon, Perris & Youdale, 1969). Furthermore, it appears that natural modulations in plasma calcium concentration control the rate of cell division when there is a physiological requirement for increased cell proliferation. Thus, when the rat is growing rapidly, the level of ionized calcium in the blood parallels both the increase in mitotic activity in bone marrow and thymus and the growth rate (Perris, Whitfield & Tolg, 1968). After haemorrhage there are also significant changes in plasma calcium levels; the animals become hypercalcaemic and both increased cell division in the bone marrow and heightened erythropoiesis result (Perris, MacManus, Whitfield & Weiss, 1971).

When investigations revealed that in other heightened and depressed erythropoietic circumstances there were also parallel changes in plasma calcium concentrations (Perris, 1971), it appeared possible that the circulating levels of erythropoietin might in some way affect the calcium homeostatic machinery of these animals. The present study, in fact, demonstrates that in addition to its known ability to increase the syntheses of RNA, haemoglobin and stromal components, and to enhance the

uptake of iron into erythrocyte precursors (see Goldwasser, 1966, and Krantz & Jacobson, 1970, for reviews), erythropoietin can induce hypercalcaemia in the rat. These changes are probably important in determining the enhanced erythropoiesis after natural or artificial increases in the level of erythropoietin. The presence of the parathyroid gland is essential for the manifestation of these new properties of the hormone.

MATERIALS AND METHODS

Different amounts of Step I (either 0·4 or 0·6 units/mg) or Step III (3·5 units/mg) ovine erythropoietin (obtained from Connaught Laboratories, Willowdale, Ontario, Canada) were dissolved in 0·9 % NaCl solution and a single injection was given subcutaneously in the dorso-thoracic region to male albino rats, of either the Sprague–Dawley or Wistar strains, weighing between 180 and 220 g. No significant differences in the responses of these two strains of rat to erythropoietin were noted. At different times after this injection plasma calcium levels and mitotic activity in the bone marrow were measured. Total plasma calcium levels were measured using the technique of Copp, Cheney & Stokoe (1963) and ionized calcium levels were determined using the Orion specific calcium ion 'flow-through' electrode system (Orion Research Inc., Cambridge, Massachusetts, U.S.A.).

Mitotic activity was normally assessed over a 6-h period by trapping cells at metaphase as they progressed through the mitotic sequence, using the metaphase-arresting agent demecolcine (Colcemid, CIBA Ltd). Suspensions and smears of bone marrow cells were prepared, fixed and stained as previously described (Perris *et al.* 1967), and the percentage of nucleated cells in the population arrested at metaphase was scored. In some experiments calcium concentrations and mitotic activity were assessed simultaneously in rats not treated with Colcemid. In this case the percentage of nucleated cells in the bone marrow population in any stage of mitosis (from late prophase to telophase) was scored. In every case at least 2000 cells per slide were counted.

When the effect of erythropoietin was tested in aparathyroid animals, the injection procedures outlined above were again followed. Rats were either parathyroidectomized by cautery, or surgically thyroparathyroidectomized as described by Ingle & Griffith (1942). Sham operations were conducted in similar fashion, except that the glands in question were not removed. All operated animals were maintained on their normal diet with tap water *ad libitum* for the first 2 days after the operation, and then transferred to a calcium deficient diet (Kenny & Munson, 1959) with free access to distilled water for the remainder of the experiment. Only parathyroidectomized or thyroparathyroidectomized animals whose total plasma calcium concentration 4 days after the operation was less than 7·5 mg/100 ml plasma were used for subsequent experiments. Erythropoietin was administered 5 days after these various operations.

To test whether the ability of erythropoietin to increase plasma calcium concentrations was due to some specific activity of the erythropoietin molecule or to some impurity in the preparation, the molecule was specifically inactivated by mild acid hydrolysis according to the technique of Rambach, Shaw, Cooper & Alt (1958). Erythropoietin was dissolved in either 5 ml distilled water or 5 ml 0·005 M-H_2SO_4 and incubated at 80 °C for 1 h. The treated erythropoietin was subsequently dialysed

against 2 l distilled water (changed twice daily) for 4 days. The resultant protein precipitate in the dialysis bag was redissolved in water and lyophilized. The lyophilized powder was subsequently dissolved in 0·5 ml 0·9% NaCl solution before injection into animals as described above. As an additional control some samples of erythropoietin were dissolved in cold water and immediately lyophilized without subsequent heating or dialysis.

In a few instances the changes in calcium concentration and bone marrow cell division induced by exogenous erythropoietin were compared with those caused by endogenous erythropoietin. To increase circulating levels of the latter, 5 ml blood were removed from normal and aparathyroid rats by cardiac puncture under light ether anaesthesia; 24 h later calcium levels and mitosis were measured as described above.

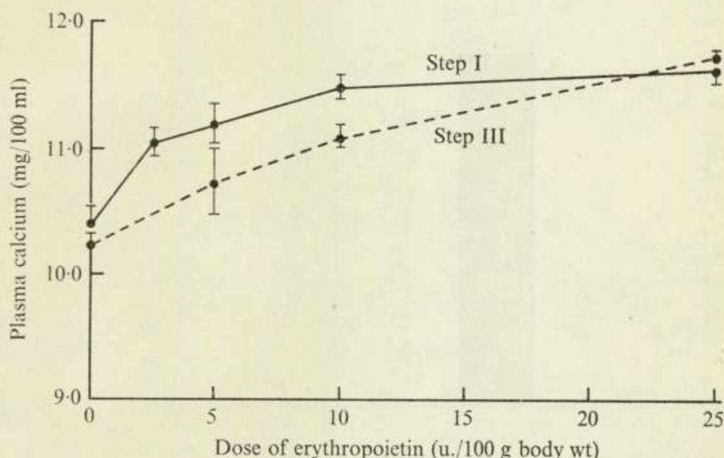

Fig. 1. Effect of erythropoietin (EPO) on plasma calcium concentrations in rats. Plasma levels were measured 24 h after single s.c. injections of Step I or Step III EPO in 0·5 ml 0·9% NaCl solution. Controls received an equal volume of saline s.c. All doses of Step I EPO caused a significant increase of calcium levels ($P < 0·001$). The Step III preparation gave significant calcium increases at the 5 ($P < 0·05$), 10 and 25 u./100 g body weight ($P < 0·001$) doses. The increases in plasma calcium concentrations above control values induced by the same doses of Step I and Step III EPO were not significantly different ($P > 0·2$ in all cases). Values are means ±S.E.M. derived from 5–28 rats in each case.

RESULTS

When increasing doses of Step I erythropoietin were administered, plasma calcium concentrations showed a progressive increase 1 day later (Fig. 1). The increment in plasma calcium concentration was maximal and of the order of 1 mg/100 ml plasma when between 10 and 20 units per 100 g body weight were given. Approximately half of this calcium increment was in the physiologically significant ionized calcium fraction (values not shown in figure). When the more highly purified erythropoietin preparation (Step III) was given, a similar effect on plasma calcium concentration was noted at all dose levels tested. This suggested that the hypercalcaemic response was probably not due to an impurity in the erythropoietin preparation (Fig. 1). This conclusion was reinforced by the experiments designed to inactivate specifically the erythropoietin molecule. Dissolution of the preparation in cold water followed by

immediate lyophilization had no effect on its ability to cause hypercalcaemia (compare Figs 1 and 2). A 1-h incubation at 80 °C after dissolution in water followed by dialysis likewise caused only a slight reduction in calcium-increasing ability. In marked contrast the mild acid hydrolysis at 80 °C completely abolished the increment in calcium concentration 24 h after the erythropoietin injection (Fig. 2). Since other workers (Rambach *et al.* 1958) have used mild hydrolysis to remove a sialic acid moiety from the molecule and thus destroy its ability to increase the incorporation of radioactive iron into erythrocyte precursors, the present findings strongly imply that the ability to increase plasma calcium concentrations is thus a specific property of the erythropoietin molecule.

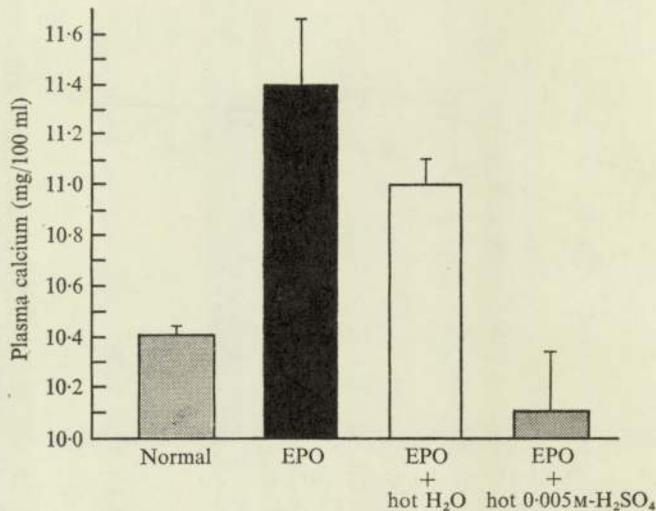

Fig. 2. Effect of mild acid hydrolysis on the ability of erythropoietin (EPO) to cause hypercalcaemia in rats. Quantities of Step I EPO were incubated at 80 °C for 1 h in either water or dilute H_2SO_4 as described under Methods. Fractions of such preparations which before treatment contained 20 u. EPO were dissolved in 0·5 ml 0·9 % NaCl solution and given as single s.c. injections to rats. Plasma calcium concentrations were measured 24 h later. Normal EPO produced a significant hypercalcaemia ($P < 0{\cdot}01$). Hot water incubation did not significantly decrease the ability of EPO to increase plasma calcium ($P > 0{\cdot}1$). Mild acid hydrolysis abolished this ability (compare Normal and Normal + hydrolysed EPO columns; $P > 0{\cdot}2$). Columns are means ± S.E.M. from four rats in each case.

In parallel with the erythropoietin-induced hypercalcaemia, mitotic activity in the bone marrow was increased (Fig. 3). This significant ($P < 0{\cdot}001$) increment in mitotic activity 1 day after administration of 10 units erythropoietin/100 g body weight (Fig. 3), was of the same order of magnitude as that produced by parathyroid hormone-induced hypercalcaemia (Perris *et al.* 1967, 1971; Rixon, 1968). In contrast, when erythropoietin was given to aparathyroid rats there were no significant changes in plasma calcium concentrations 1 day later (Fig. 4). Erythropoietin also failed to stimulate bone marrow mitosis significantly in the aparathyroid rats (Table 1). Similarly haemorrhage, which causes endogenous erythropoietin release and which has been shown to increase plasma calcium levels in normal rats (Table 1 and Perris *et al.* 1971), also failed to provoke hypercalcaemia or increased proliferation in the

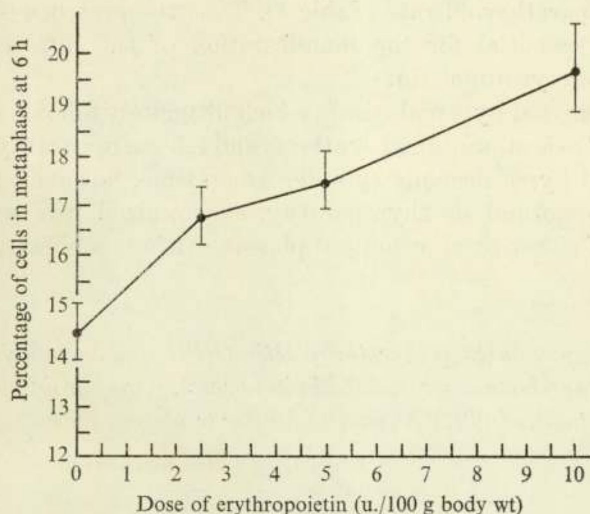

Fig. 3. Effect of Step I erythropoietin (EPO) on bone marrow mitosis of rats. Twenty-four hours after subcutaneous injection of EPO, mitosis was significantly increased at all doses tested. The level of significance increased from $P < 0{\cdot}05$ to $P < 0{\cdot}001$ with increasing dose. Values are means $\pm$S.E.M. from 9 to 20 rats in each case.

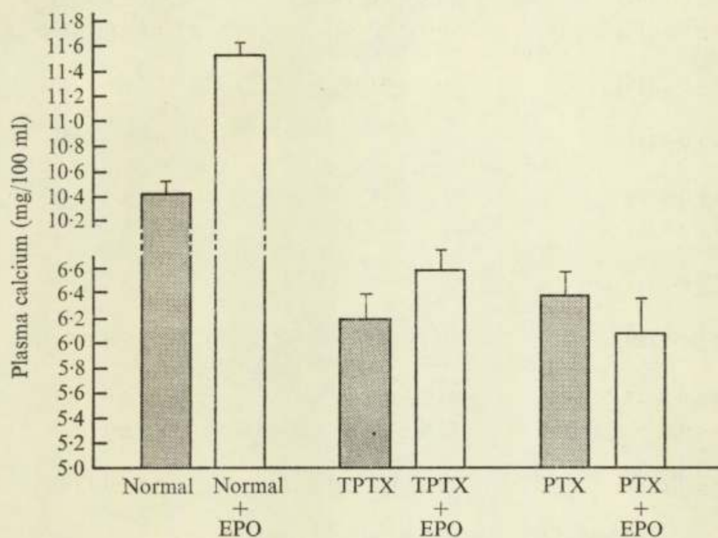

Fig. 4. Effect of Step I erythropoietin (EPO) on plasma calcium concentrations in normal, thyroparathyroidectomized (TPTX) and parathyroidectomized (PTX) rats. Concentrations were measured 24 h after subcutaneous injection. EPO had no significant effect on calcium concentrations in thyroparathyroidectomized or parathyroidectomized rats ($P > 0{\cdot}05$ and $P > 0{\cdot}2$ respectively) but produced a significant hypercalcaemia in the intact rat ($P < 0{\cdot}001$). Values are means (columns) + S.E.M. (vertical line with horizontal bar) from 6 to 28 animals in each case.

bone marrow of the aparathyroid rats (Table 1). Thus the presence of a functional parathyroid gland is essential for the manifestation of the hypercalcaemic and mitogenic properties of erythropoietin.

The parathyroid-mediated hypercalcaemia which ultimately follows erythropoietin injection might be due to a supranormal synthesis and release of parathyroid hormone provoked by an initial hypocalcaemic episode. At no time, however, after erythropoietin injection into normal or thyroparathyroidectomized rats was there any significant lowering of either total or ionized plasma calcium concentrations (Fig. 5, Table 2).

Table 1. *The effect of exogenous (Step I) erythropoietin (EPO) and haemorrhage on plasma calcium concentrations and bone marrow mitosis in normal, parathyroidectomized (PTX) and thyroparathyroidectomized (TPTX) rats 24 h after treatment (means* ± S.E.M.)

			Bone marrow mitosis			
Treatment	Plasma calcium (mg/100 ml)	*P*	Per cent nucleated cells in metaphase 6 h after Colcemid	*P*	Per cent nucleated cells in mitosis	*P*
Normal + saline	10·4 ± 0·1 (28)	< 0·001	14·5 ± 0·6 (20)	< 0·001	1·2 ± 0·1 (14)	< 0·01
Normal + EPO	11·5 ± 0·1 (22)		19·7 ± 0·9 (20)		1·8 ± 0·1 (8)	
Normal + sham bleeding	10·2 ± 0·1 (17)	< 0·01	16·7 ± 1·3 (6)	< 0·05	1·2 ± 0·1 (13)	< 0·01
Normal + 5 ml haemorrhage	11·2 ± 0·1 (15)		20·7 ± 1·1 (6)		1·7 ± 0·1 (15)	
PTX + saline	6·8 ± 0·5 (4)	NS	—	—	0·9 ± 0·1 (3)	NS
PTX + EPO	6·3 ± 0·6 (5)		—		0·9 ± 0·1 (3)	
PTX + sham bleeding	6·8 ± 0·7 (5)	NS	—	—	—	—
PTX + 5 ml haemorrhage	6·0 ± 0·3 (4)		—		—	
TPTX + saline	6·2 ± 0·2 (20)	NS	9·7 ± 0·6 (5)	NS	1·0 ± 0·1 (5)	NS
TPTX + EPO	6·6 ± 0·2 (18)		11·5 ± 0·6 (5)		1·1 ± 0·2 (5)	
TPTX + sham bleeding	5·6 ± 0·2 (8)	NS	12·6 ± 1·4 (7)	NS	0·8 ± 0·1 (7)	NS
TPTX + 5 ml haemorrhage	5·2 ± 0·1 (8)		12·2 ± 0·8 (7)		0·9 ± 0·1 (6)	

Numbers of animals used are given in parentheses.
NS = not significant ($P > 0·05$).

DISCUSSION

The demonstration that erythropoietin can increase plasma calcium concentrations and thereby enhance bone marrow cell proliferation in rats with intact parathyroid glands (Figs. 1–5, Table 1) can now be added to other studies implicating the parathyroid in the physiological control of bone marrow cell division and erythropoiesis (Perris, 1971; Perris & Whitfield, 1971; Perris *et al.* 1971). When erythropoiesis and circulating erythropoietin levels are increased after haemorrhage, during pregnancy,

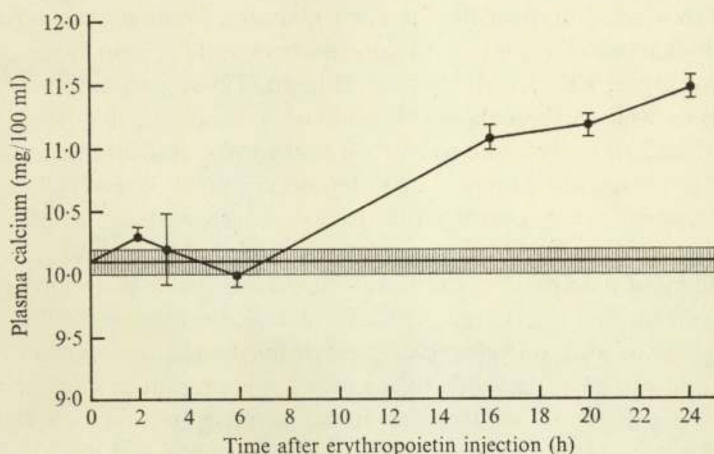

Fig. 5. Effect of Step I erythropoietin (EPO) on plasma calcium concentrations in rats. At 2, 3 and 6 h after s.c. injection EPO produced no significant changes in calcium levels compared with saline-injected controls ($P > 0{\cdot}1$). Values at later time periods were significantly higher than normal ($P < 0{\cdot}01$). The horizontal line and shaded area represent the mean plasma calcium concentration of 24 control rats ± 1 S.E.M. Points are means ± S.E.M. derived from between 5 and 14 rats in each case.

Table 2. *The effect of Step I erythropoietin (EPO) on plasma calcium levels immediately after injection in normal and thyroparathyroidectomized (TPTX) rats (means* ± S.E.M.)

Time after EPO injection (h)	Plasma calcium in normal rats (mg/100 ml)		Plasma calcium in TPTX rats (mg/100 ml)	
	Total	Ionized	Total	Ionized
0	10·1 ± 0·1 (24)	5·3 ± 0·1 (16)	5·0 ± 0·2 (4)	2·1 ± 0·1 (6)
3	10·2 ± 0·3 (5)	5·4 ± 0·1 (5)	5·0 ± 0·2 (8)	2·1 ± 0·1 (6)
6	10·0 ± 0·1 (14)	5·0 ± 0·2 (10)	5·0 ± 0·3 (4)	2·1 ± 0·1 (6)

Step I erythropoietin in 0·5 ml 0·9 % NaCl solution (10 u./100 g body weight) was injected subcutaneously into normal and TPTX rats. At no subsequent time did calcium levels in normal and TPTX rats change significantly ($P > 0{\cdot}1$). Number of animals is given in parentheses.

or after cobaltous chloride treatment (Contopoulos, Van Dyke & Simpson, 1956; Jacobson & Goldwasser, 1958; Jepson & Lowenstein, 1968) rat plasma calcium concentrations and bone marrow mitosis increase in parallel (Perris, 1971; Perris *et al.* 1971). In contrast, during transfusion-induced polycythaemia when erythropoietin levels are presumably negligible or very low (Adamson & Finch, 1968) calcium levels are decreased as is bone marrow proliferation (Perris, 1971). Similar mechanisms may exist in man. The heightened erythropoiesis which presumably accompanies and counteracts the inadequate circulation and oxygen supply to the tissues in children with cardiac defects is accompanied by increased ionized calcium concentrations in the plasma (Putman, 1972). The present study suggests that the different circulating levels of erythropoietin in the plasma are directly responsible, via the parathyroid gland, for the calcium-induced mitotic changes in these various heightened and depressed erythropoietic circumstances.

Although to our knowledge there is no evidence to suggest that derangements in calcium homeostasis lead to clinical abnormalities in marrow cellularity, this may be

because other factors will compensate for such changes. Over a long-term period erythropoietin, which has a direct effect on bone marrow cell division (Kuna, Gordon, Morse, Lane & Charipper, 1959; Matoth & Kaufmann, 1962; Reissmann & Samorapoompichit, 1970) as well as the calcium-dependent indirect effect described in the present study, will undoubtedly serve to control erythroid cellularity and circulating red cell numbers. However, when there is a sudden requirement for red cell production such as follows haemorrhage, a parathyroid-mediated hypercalcaemia plays a vital role in the rapid restoration of red cell mass (Perris *et al.* 1971).

The general ability of calcium to stimulate cell division in a wide variety of cell types is now well established (see Perris, 1971, for detailed references). This property is shared by a number of hormones (such as growth hormone, neurohormones, parathyroid hormone and prolactin) provided that calcium is present in the extracellular environment. These calcium-dependent hormones appear to sensitize the cell to calcium which initiates a chain of events culminating in mitosis (Whitfield, Perris & Youdale, 1969). By means of a cyclic 3′,5′-adenosine monophosphate increment within the cell (MacManus, Whitfield & Youdale, 1971; MacManus, Perris, Whitfield & Rixon, 1972) calcium and calcium-dependent hormones first induce the initiation of DNA synthesis within the cell nucleus; this is followed by the entry of these stimulated cells into division a few hours later (MacManus & Whitfield, 1969*a*, *b*; Whitfield *et al.* 1969; MacManus & Whitfield, 1970; Whitfield, MacManus & Gillan, 1970*a*, *b*; Whitfield, MacManus & Rixon, 1970*a*, *b*). Presumably the parathyroid-mediated hypercalcaemia which was induced by erythropoietin (Figs 1, 4 and 5, and Table 1) stimulated bone marrow cell division in a similar fashion. The means whereby erythropoietin interacts with the calcium homeostatic machinery to cause hypercalcaemia is less clear however.

The normal stimulus for increased parathyroid hormone synthesis and release is a lowering of plasma calcium concentrations (Copp & Davidson, 1961; Talmage & Toft, 1961; Sherwood, Mayer, Ramberg, Kronfeld, Aurbach & Potts, 1968). If erythropoietin could in some way cause an initial hypocalcaemic episode this might serve to increase circulating parathyroid hormone levels; a sustained hypercalcaemia has been observed to follow a hypocalcaemic phase in the bled rat (Perris *et al.* 1971). How such a protracted hypercalcaemia can be reconciled with the normal calcium homeostatic system is not clear. In contrast to the erythropoietic stimulus of haemorrhage, exogenous erythropoietin did not cause any similar initial lowering in plasma calcium concentrations in either normal or parathyroidectomized rats (Table 2, Fig. 5).

If erythropoietin were directly parathyrotrophic this would account for the increased plasma calcium concentrations observed after erythropoietin injection (Figs. 1, 2, 4 and 5, and Table 1), and for posthaemorrhagic hypercalcaemia (Table 1 and Perris *et al.* 1971); in the latter case endogenous erythropoietin levels are supranormal (Jacobson & Goldwasser, 1958; Fried, Johnson & Heller, 1970). An alternative mechanism for erythropoietin-induced hypercalcaemia might involve a sensitization by erythropoietin of the target organs (bone, small intestine or kidney) to parathyroid hormone. By this means a normal circulating level of parathyroid hormone might cause a greater than normal degree of bone resorption, intestinal absorption or tubular reabsorption of calcium. At the concentrations used in the present study erythropoietin clearly does not stimulate these processes directly for no significant increase

in calcium concentrations was noted after erythropoietin administration to aparathyroid rats (Fig. 4, Table 1). However, erythropoietin could act synergistically with parathyroid hormone in the normal animal, or exercise a permissive role for parathyroid hormone-stimulated processes. Vitamin D and its metabolites and a number of other stimulators of bone resorption seem to have this type of action (Blunt, Tanaka & DeLuca, 1968; Raisz, 1970). Whatever the mechanism, it is now quite clear that there is an important involvement of the parathyroid gland in the action of erythropoietin and in erythropoiesis in the whole animal.

A number of authors (Gallien-Lartigue & Goldwasser, 1964; Dukes & Goldwasser, 1965; Krantz & Goldwasser, 1965; Pieber-Peretta, Rudolph, Hodgson & Peretta, 1965; Hrinda & Goldwasser, 1966; Gross & Goldwasser, 1970; Ortega & Dukes, 1970) have demonstrated that the initial or primary actions of erythropoietin on bone marrow cells are to stimulate the syntheses of RNA, haemoglobin and stromal components and to facilitate the incorporation of iron into red cell precursors, thus promoting the differentiation of primitive stem cells into erythroblasts. The stimulation of DNA synthesis and cell division is not necessary for the expression of these properties of erythropoietin (Erslev & Hughes, 1960; Gallien-Lartigue & Goldwasser, 1964; Dukes & Goldwasser, 1965; Gross & Goldwasser, 1970; Ortega & Dukes, 1970). However, erythropoietin undoubtedly does stimulate DNA synthesis and proliferation, albeit at a later stage than the maturation effects mentioned above are manifest (Kuna *et al.* 1959; Matoth & Kaufmann, 1962; Gross & Goldwasser, 1970; Ortega & Dukes, 1970; Reissmann & Samorapoompichit, 1970; Perris & Whitfield, 1971, and Fig. 3). Erythropoietin-induced and -controlled proliferation and maturation occur independently, but in parallel, to ultimately produce increased numbers of circulating erythrocytes.

Although erythropoietin can increase cell division in isolated bone marrow cells (Kuna *et al.* 1959; Matoth & Kaufmann, 1962) the parathyroid-mediated hypercalcaemia caused by erythropoietin in the whole animal (Figs 1, 2, 4 and 5, and Table 1) will amplify such an effect. By speeding up the division of cells in the bone marrow, the hypercalcaemia may not only provide a larger base of cells upon which erythropoietin could subsequently act as a differentiating agent, but also may hasten the appearance of erythrocytes in the peripheral blood. The parathyroid gland must now be considered as an important additional vector in the control of erythropoiesis in the whole animal.

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