# Some Aspects of the Biochemistry of Oestrogens

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Summary:

The thesis is concerned with several aspects of oestrogen biochemistry. It has two main divisions. In the first part, a review of the clinical and physiological rôle of oestrogens is given, together with a discussion of the use to which oestrogen determinations are put in the management of pregnancy. The methods currently available for the determination of oestrogens in body fluids are reviewed and criticised. The available methods are found to be inadequate for a variety of reasons, and a new assay for oestrogens in pregnancy urine is described which overcomes these difficulties. This assay is based on the native fluorescence of the oestrogens, and the theoretical and practical basis for this method of assay is presented and discussed. A certain amount of clinical evidence is given to support the suggestion that this test is better suited to the hospital environment than any previously published method.

The second part of the thesis concerns the effects which the oestrogen molecule may have on the cell membranes of the tissues with which it comes into contact. The numerous effects which oestrogens appear to have on tissues which are not considered to be target tissues suggest that there is more than one mode of action for the oestrogen response. This problem is investigated in the highly responsive cell type, the thymic lymphocyte, where oestrogen is found to inter-

-fere with the calcium mediated responses consonant with stimulation by mitogens or hormones. The method is also shown to be applicable to study of the highly specialised cell remnant, the blood platelet.

A mechanism is proposed whereby oestrogen may interfere with the hormonal response of cells within the body, and the relationship between this effect and the rôles of ions and cyclic nucleotides within the cell is discussed.

These propositions are based on the data obtained with the use of a new series of fluorescent dyes which are used as probes for membrane characteristics such as the trans-membrane potential. Experimental detail is given where appropriate, and the techniques used are described in detail.

The conclusion of the thesis is that oestrogens may affect cells which have hitherto not been con-sidered to be particularly responsive to the hormone, and that this effect of oestrogens may well lie in the ability of the hormone to interfere with ion movements across, and within, the plasma membrane. It may prove to be that the same structural characteristic of the oestrogen molecule which allows for a fluorescent determination of the hormone also permits the inter-vention of the hormone in the processes of cells other than classical oestrogen target cells.

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It is the mark of an educated mind to expect that amount of exactness in each kind which the nature of the particular subject admits.

Aristotle (Nicomachean Ethics, Book 1, 1094, b25)

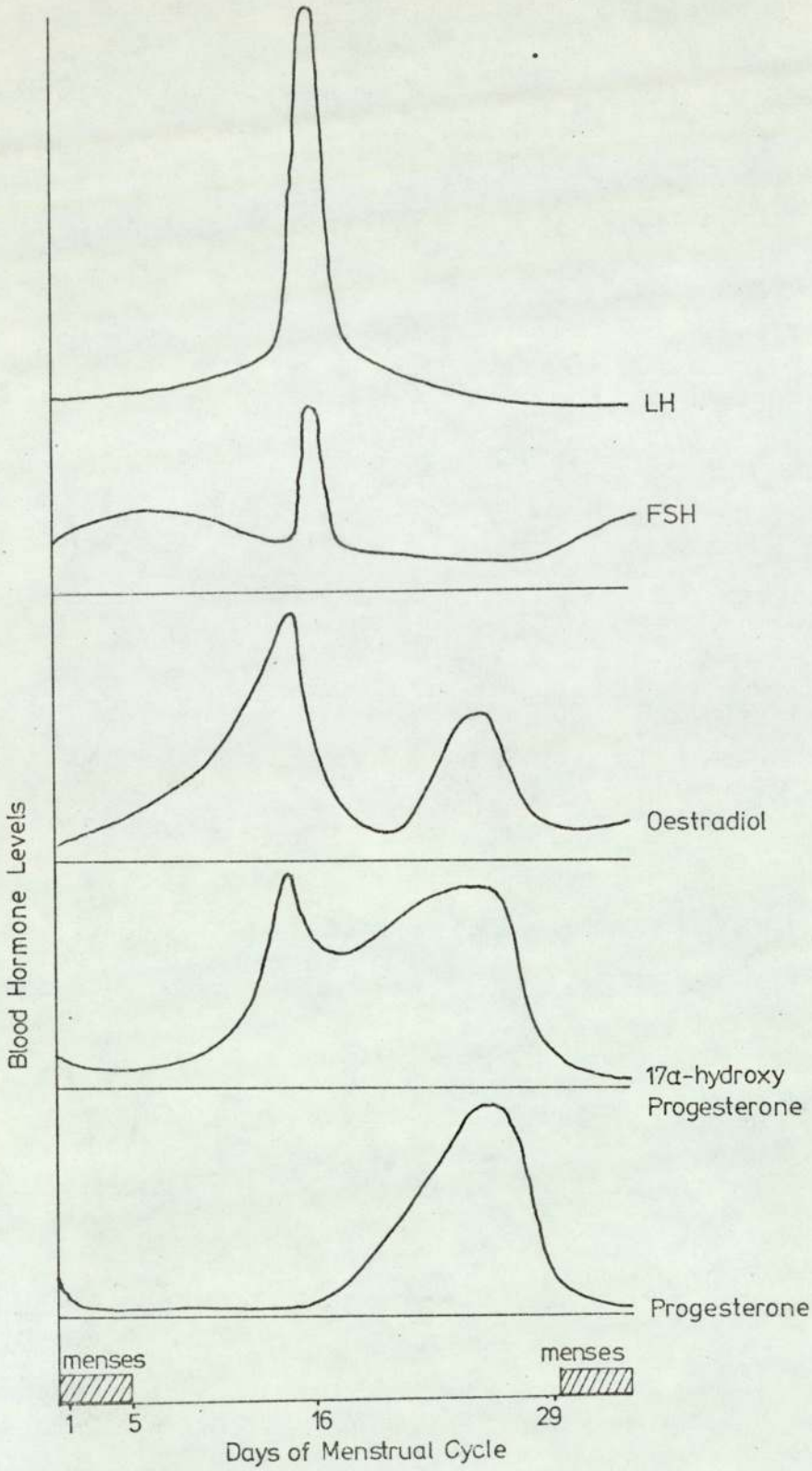



Fig.1: To show the relative concentrations of plasma hormones at different stages of the menstrual cycle.

## I Hormones of the Menstrual Cycle

Women, unlike the females of many other species, do not display periodic oestrus, this lack possibly being the product of social suppression during the course of human evolution. They do show, however, a periodic blood-stained vaginal discharge (the menses) which occurs, typically, at monthly intervals. It used to be thought that menstruation was a form of oestrus, but this is not now held to be so.

Menstruation lasts about 4 or 5 days, and marks the end of the functional life of the corpus luteum; the first day of menstruation is regarded as day 1 of a new cycle. This monthly cycling is primarily under humoral control, but higher centres of the brain can also exert a profound effect on the timing of ovulation and the menses. For instance, the act of sexual intercourse may precipitate unscheduled ovulation, and severe emotional stress or anxiety can arrest the menstrual cycle, sometimes for a period of years[378]. However, if we look at some of the hormone changes which occur during the menstrual cycle (Fig.1) we see that at the beginning of the follicular phase the levels of follicle stimulating hormone (FSH) and luteinising hormone (LH) are rising. As a result of this, a Graafian follicle is allowed to develop in one of the ovaries and its theca interna cells secrete an increasing amount of oestradiol-17B, androstenedione, and

17a-hydroxyprogesterone. The rising oestrogen secretion before ovulation begins to depress the secretion of FSH, and at the same time it causes the uterine glands to proliferate[396]. Eventually, the oestrogen triggers off an ovulatory discharge of LH from the pituitary,[104] usually accompanied by a simul--taneous discharge of FSH, but the time intervals between the oestrogen peak, the LH peak and the moment of ovulation are not known with any degree of precision. After ovulation, a corpus luteum begins to form and secrete large amounts of progesterone. It also secretes some 17a-hydroxyprogesterone and oestradiol-17β, so that the levels of all three steroids are elevated in the luteal or secretory phase of the cycle[396].

The life of the corpus luteum is not known with certainty, it may vary from 7 to 14 days from woman to woman, and we still do not have much idea of which pituitary gonadotrophins are necessary for the maintainance of the corpus. LH is certainly involved since it can stimulate progesterone secretion and prolong luteal life. But other factors must also be implicated because continuous treatment with LH or human chorionic gonadotrophin (HCG) can only prolong luteal life by a few days, and the corpus luteum will still regress during the course of the treatment. Some workers have suggested that prolactin may be a key component of the pituitary luteotrophic stimulus, and its role, like that of

-F2a prostaglandin (which is present in the uterus at the end of the menstrual cycle) remains shrouded in doubt.

Once the corpus luteum has started to regress the hypertrophied secretory endometrium is suddenly deprived of its hormonal support; the endometrial arteries go into spasm, and the epithelium is sloughed off into the uterine lumen, together with a certain amount of blood. The prostaglandin in menstrual fluid probably has a stimulatory action on the smooth muscle of the uterus, which may be one reason for the pains which women experience at this time. Another conse--quence of luteal regression is that the hypothalamus escapes from the inhibitory effects of progesterone and oestrogen, so that the pituitary begins to secrete more FSH and LH in preparation for the next cycle[378].

Thus, although it has long been assumed that the pituitary (or placental) gonadotrophins play the major role in concerting ovarian synthesis and the secretion of hormones such as oestrogen, their precise role has always been obscure. More recently their relative importance has been brought into a truer perspective to show a more restrained role for these trophic hormones in the elaboration and release of the steroid hormones involved in the menstrual cycle.[104,185] Whereas, as noted above, the direct secretion-regu--lation factors are still unknown, recent work suggests that it may be the nucleic acids of ovarian cells themselves which ultimately programme the pattern of

steroid synthesis. For example, RNA extracted from one endocrine gland may impose its own synthetic pattern on another gland when incubated with it *in vitro*[425], and this may well be relevant to the *in vivo* situation. Regulation of the menstrual cycle thus depends on the interaction of specific intracellular determinants with the more generalised, and non-specific, stimulation by gonadotrophins (or their mediators such as cAMP).

## II Hormones in Pregnancy

In addition to governing the menstrual cycle, hormones also have a major part to play in the maintainance of pregnancy; their role becoming prominent with the evolution of vivipary when a series of innovations first appeared such as a reduction in the yolk content of eggs, formation of the placenta and retention of the young within the female genital tract. Endocrine control is exerted primarily by the hormones of the pituitary, the ovary and the placenta.[349]

A characteristic feature of early pregnancy is the presence of a corpus luteum actively secreting progesterone, and a requirement of pregnancy is that the luteal phase of the oestrus cycle should be prolonged and ovulation suppressed. However, the important question of how a woman 'knows' that she is pregnant, and how she prolongs her oestrus cycle and delays the recurrence of ovulation cannot yet be satisfactorarily answered. It is thought that in

women the pregnancy is probably recognised by the luteotrophic activity of the conceptus, which prolongs the life of the corpus luteum and delays menstruation, but the position is confused, and it is certain that there is not a single mechanism for the recognition of pregnancy that is common to all species.

The corpus luteum of pregnancy is composed of morphologically distinct cells, large and round in appearance, and well endowed with a pale-staining cytoplasm. The ultrastructure of the actively secreting cell resembles that of all steroid-secreting cells: the cytoplasm being packed with smooth endoplasmic reticulum and containing lipid droplets and an enlarged Golgi complex. The lipid droplets probably represent intracellular stores of steroid precursors, depleted during maximum synthesis, as in pregnancy, and restored at times of reduced steroid production, as during luteal regression.

The steroid which is produced, by the ovary, in the greatest quantities is progesterone. It is the most active of the naturally occuring progestagens, but the total amount of progesterone stored in the corpus luteum is small and is equivalent to only a few minutes' supply.

The formation of the corpus luteum of pregnancy probably depends upon the secretion of HCG by the trophoblast within a few days of implantation, together with human chorionic somatomammotrophin (HCS). The placenta may assume some of the functions

of the pituitary and so control the corpus luteum, but in addition it may assume some of the endocrine functions of the ovary. In fact the placenta produces enough progesterone to maintain pregnancy, at a very early stage, so that the role of the corpus luteum in pregnancy maintainance is short lived.

The regulation of gestation comprises a series of complex interactions between protein hormones that are luteotrophic, and steroid hormones that are directly concerned with pregnancy maintainance. Steroids also modify the secretory activity of the pituitary, either by a negative feedback mechanism as in the inhibition of LH secretion by progesterone, or by a positive feedback, as in the stimulus of prolactin secretion by oestrogen. In striking contrast to pituitary feedback, there is no evidence of a comparable regulation of hormone production by the placenta; in fact a notable feature of the placenta is its autonomy. If the conceptus (fetus and placenta) is removed from an experimental animal the corpus luteum regresses, milk secretion may begin, and the animal may return to oestrus; but if the fetus alone is removed, and the placenta is left in situ, the period of gestation is unchanged (at least in experiments using rat, mouse, rabbit and monkey). This experimental condition is known as 'placental pregnancy'; the placenta continues to grow, the mammary glands develop and there is 'pseudo-parturition'.

The formation of large quantities of placental hormones in such close proximity to the fetus raises the question of whether they have any direct effect on the fetus itself, and of whether the fetus is protected against the high levels of these potent substances. The amounts of protein and steroid hormones stored in the placenta are generally low, and are equivalent to only a few minutes' supply. There is an effective intraplacental barrier to the transfer of protein hormones such as HCS, though small quantities of HCG may reach the fetus. In contrast there is appreciable transfer of progesterone and, to a lesser extent, of oestrogens to the fetus. These steroids are rapidly metabolised in the fetus, principally in the adrenals, liver and peripheral tissues, by hydroxylation or conjugation, mechanisms that effectively reduce the potency of the parent compound. These reactions have already been described for the non-pregnant state, and there is no reason to suggest that the nature of the protective conju--gation and hydroxylation reactions is any differeht in pregnancy. There are, of course, major differences in the relative activities of the enzymes concerned, and in the relative importance of the reactions which might be employed. It appears that the conjugation reactions are of paramount importance in protecting the developing fetus from high levels of hormones. Whilst it may be true to imply only superficial

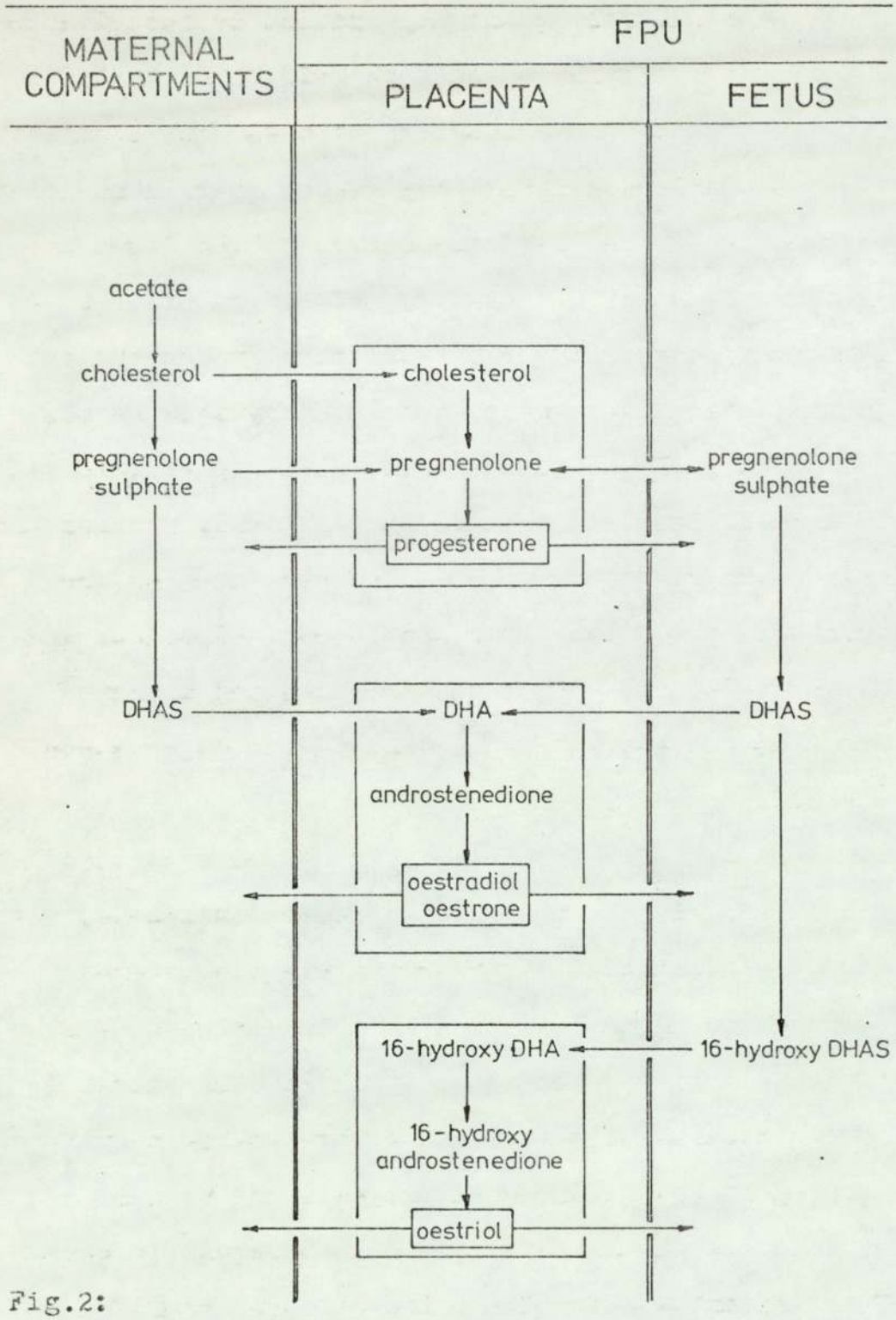



Fig.2:
Steroid synthesis by the placenta showing compartmen-
-talisation and discontinuity in the formation from
maternal or fetal precursors.

differences between the pregnant and the non-pregnant state when describing the degradation or inactivation of steroids, the same cannot be held to be true for the elaboration and anabolism of them[94]. For example, the conjugation processes may render steroid substrates accessible to specific enzyme systems localised in specific organs. For example, sulphation of oestrone enables this steroid to be specifically accumulated in the fetal liver where it is hydroxylated in the 16a position[110]. In addition, the stereospecificity of developing fetal tissues is so high that, for instance, only 3a,5B-reduction products of progesterone are formed by the fetal liver and intestinal tract, and exclusively 3B,5a-forms by the lungs and kidneys[36]. Fig.2 is one attempt[99] to integrate the major steroid anabolic routes of pregnancy.

With reference to this figure it can be seen that cholesterol is the principal precursor molecule, and most of this enters the placenta via the maternal circulation. This cholesterol is converted by the placenta into pregnenolone, which is then secreted to the maternal and fetal compartments, or converted to progesterone. Placental progesterone hardly contributes to the formation of oestrogens in the feto-placental unit. The principal precursor for these steroids is dehydroepiandrosterone sulphate (DHAS), formed in the fetal and maternal adrenals. DHAS enters the placenta from both these compartments and is converted, via androstenedione and testosterone,

into oestrone and oestradiol 17β, but not to oestriol, this latter conversion being dependent upon a 16α-hydroxylation which is performed chiefly by the fetal liver. The subsequent feto-placental metabolism of oestrogens is characterised by dynamic changes in conjugation processes rather than by changes in the steroid moiety[222].

In human pregnancy the rate of placental hormone secretion appears to be correlated with the size of the placenta, as is evident from the relation of the blood levels of total oestrogens, progesterone and HCS, and the placental weight. Other factors such as the wellbeing of the conceptus also affect these parameters, and this topic will be the subject of a later section of this thesis.

Clearly, the conceptus and the ovaries are not the sole tissues responsible for the humoral control of pregnancy, but the function of the other endocrine organs, such as the thyroid, parathyroid, pancreas and adrenals, is related to the differing conditions imposed by pregnancy rather than to the fact that they have a major role in the maintainance of gestation. The adrenals, frequently heavier in females than in males, may become more active and enlarged. Biochemically, they have the capacity to produce appreciable amounts of gonadal steroids, (oestrogens, androgens and progestogens). The concentrations of these steroids in venous adrenal blood is high under

the conditions of stress induced experimentally by surgery, anaesthesia or the administration of adreno-corticotrophic hormone (ACTH); but whether they are high in the normal, unstressed condition is open to debate. In fact, the adrenals may well have some of their functions completed for them by pregnancy-associated tissues; for example, the corpus luteum secretions may well tend to make the adrenal secretion of the same compounds redundant. Thus, during pregnancy there is a change in the balance of concentrations of many hormones which circulate in the non-pregnant female, and it is these balances which are responsible for concerting the events which make up the main features of pregnancy. For example, synergistic interactions between progesterone and oestrogens are usually encountered when the concentrations differ by about three orders in favour of progesterone[378], so as the difference in concentrations decreases, their actions become increasingly antagonistic. The histological changes that are observed in target organs during gestation are the result of the interactions between oestrogens and progesterone. This synergism causes distinctive changes in the appearance of other target organs during pregnancy, notably the mucification of the vagina, the growth of alveoli and ducts in the mammary glands and the enlargement of the myometrium of the uterus. This enlargement results primarily from the growth of existing cells rather than from

the proliferation or increase in cell numbers.

Progesterone has a multiple role in the main--tainance of gestation. It delays ovulation by the suppression of LH secretion; it acts on the endometrium to prepare for the reception of the embryo; and it renders the contractile myometrium quiescent so that implantation proceeds normally and the expulsion of the embryo is prevented. This latter property has been described as the 'progesterone block' of myometrial activity. Numerous theories have been proposed to explain the mechanism of this block. It has been suggested[83] that progesterone hyperpolarises the myometrial cell, reducing its excitability, and, consequently, a decline in progesterone levels, as at term, would facilitate myometrial excitation. Thus, changes in the ionic permeability of the membrane of the myometrial cell induced by progesterone are likely to underlie the decline in myometrial activity, and this theme will be developed, in the context of oestrogen action, in later sections of this thesis.

In contrast to progesterone, oestrogen stimulates the contractile activity of the quiescent, immature uterus, causing regular, rhythmic contractions. At, or just before, the time of parturition this activity becomes synchronised, and with the release of oxytocin the contractions increase in both frequency and amplitude. The increase in myometrial activity in late pregnancy corresponds with the time when the concentration of oestrogens in the blood reaches its

highest levels, and it appears that the removal of the progesterone block towards the end of gestation may be related not only to a reduction in the local concentration of progesterone but also to the antag--onism of a raised oestrogen level. Clearly, these relative concentrations are of vital importance. The amounts of hormones in blood reflect the secretory activity of the endocrine organs (more closely than urinary levels of hormone metabolites) and, what is more important, they reflect the concentration of hormones transported to target tissues such as the uterus and mammary gland.

Because of their high lipid solubilities, oestrogens and progesterone are transported in blood largely bound to plasma proteins. Some proteins, such as albumin, have a very large capacity for steroid binding but their affinity is low; others such as transcortin (corticosteroid-binding globulin) have a low capacity but a high affinity. In consequence, only a small proportion of the oestrogen and progesterone measured in blood is present in a form that is free, or non-protein bound. The presence of plasma proteins in gestation with high affinities for steroid hormones affects the rate of metabolism of a steroid and its concentration in blood, and also its availability to target tissues. A more detailed expansion of the role of sex-hormone-binding globulin (SHBG) will be given in the next section.

## III Oestrogen Metabolism

All substances producing oestrus and vaginal corni-
-fication in spayed mice or rats are said to be oestrogenic substances or 'oestrogens'. Although they need not be steroids, in popular use, the term oestrogen has come to be closely associated with the three principal steroidal oestrogens which are to be found in the plasma of healthy mammals shown in Fig. 3 below:


Fig. 3:

Although the oestrogens play a profound role in the humoral homeostatic control systems of both male and female animals, this section will concentrate on the metabolism of steroidal oestrogens in the human female - this being the most pertinent to the later discussions.

The oestrogenic (or follicular) hormones are C-18 steroids, and the theca interna of the developing graafian follicle appears to be the main site of production, although appreciable oestrogen synthesis occurs in the placenta and the adrenal as well as the ovary. (In the male, the testes are a site of production too).

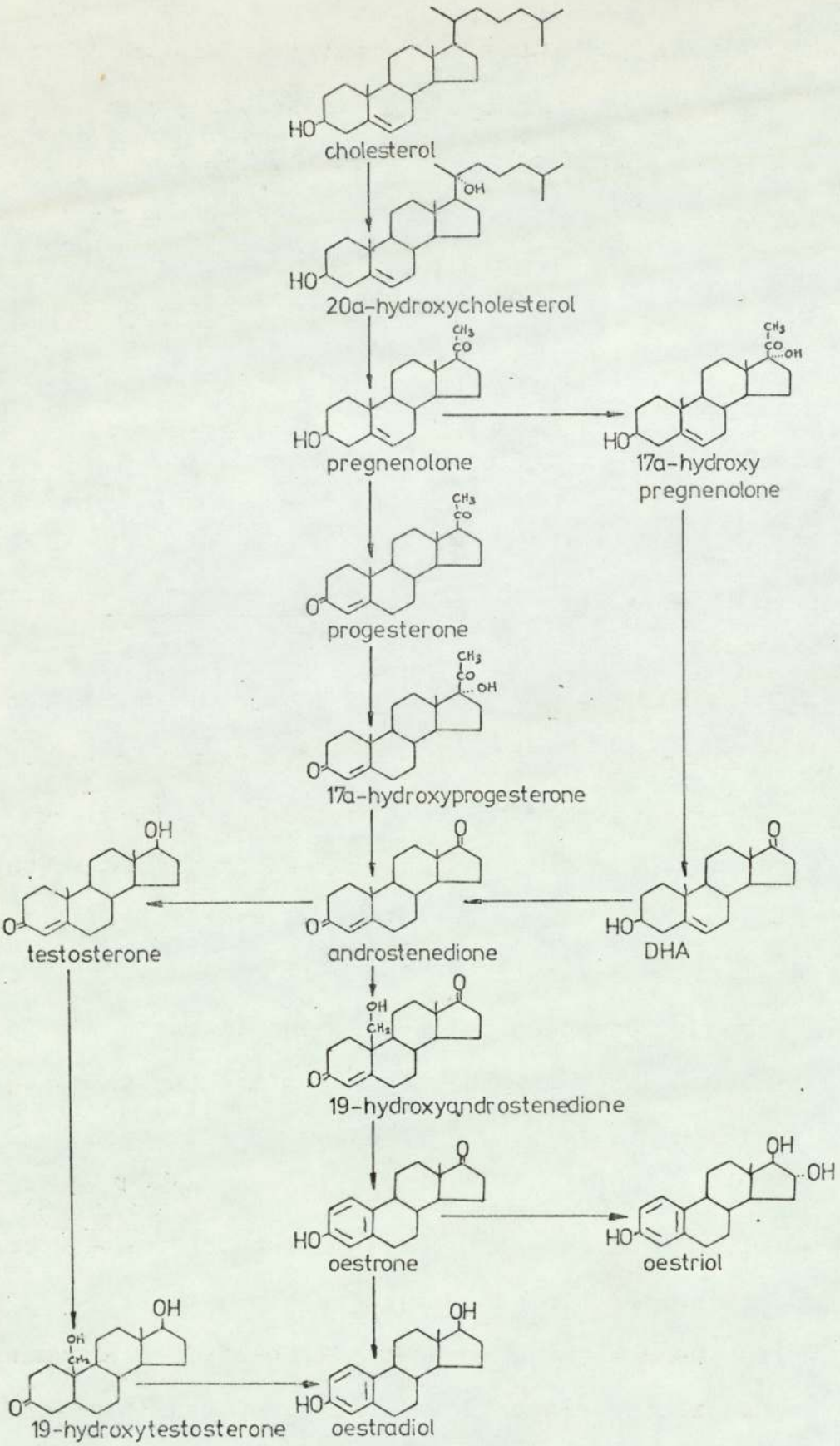



FIG.4: Anabolism of oestrogens in women

The principal oestrogenic hormone in the ovary is oestradiol, whereas oestrone is probably the circulating oestrogenic hormone[423]. Oestrone and oestradiol are interconvertible, and they exist in the ovary in a state of equilibrium with each other. Oestriol predominates in the circulation of pregnant women, and is produced from oestrone by hydroxylation at C-16 and reduction of the ketone group at C-17.

Cholesterol is the starting point of oestrone biosynthesis in the ovary, and although the exact mechanism of its synthesis is not known, it is highly probable that progesterone and testosterone are immediate precursors. This is supported by the fact that placental tissue can convert testosterone to androstenedione, which, in turn, is converted to oestrone. Ovarian tissue also forms androstenedione from testosterone as well as from progesterone. It is suggested that the degradation of the androgens involves hydroxylation of the methyl group at C-19, followed by the removal of the entire resultant primary alcohol group to produce a C-18 steroid with an aromatic nucleus characteristic of the oestrogens. A scheme for the synthesis of the steroidal oestrogens is shown in Figs.4 & 5.

Cholesterol levels are maintained at a remarkably constant level in the circulating blood. The major tissue of synthesis is the liver, more specifically the krypts of Lieberkuhn, and its production is subject to control, (either by feed-back inhibition

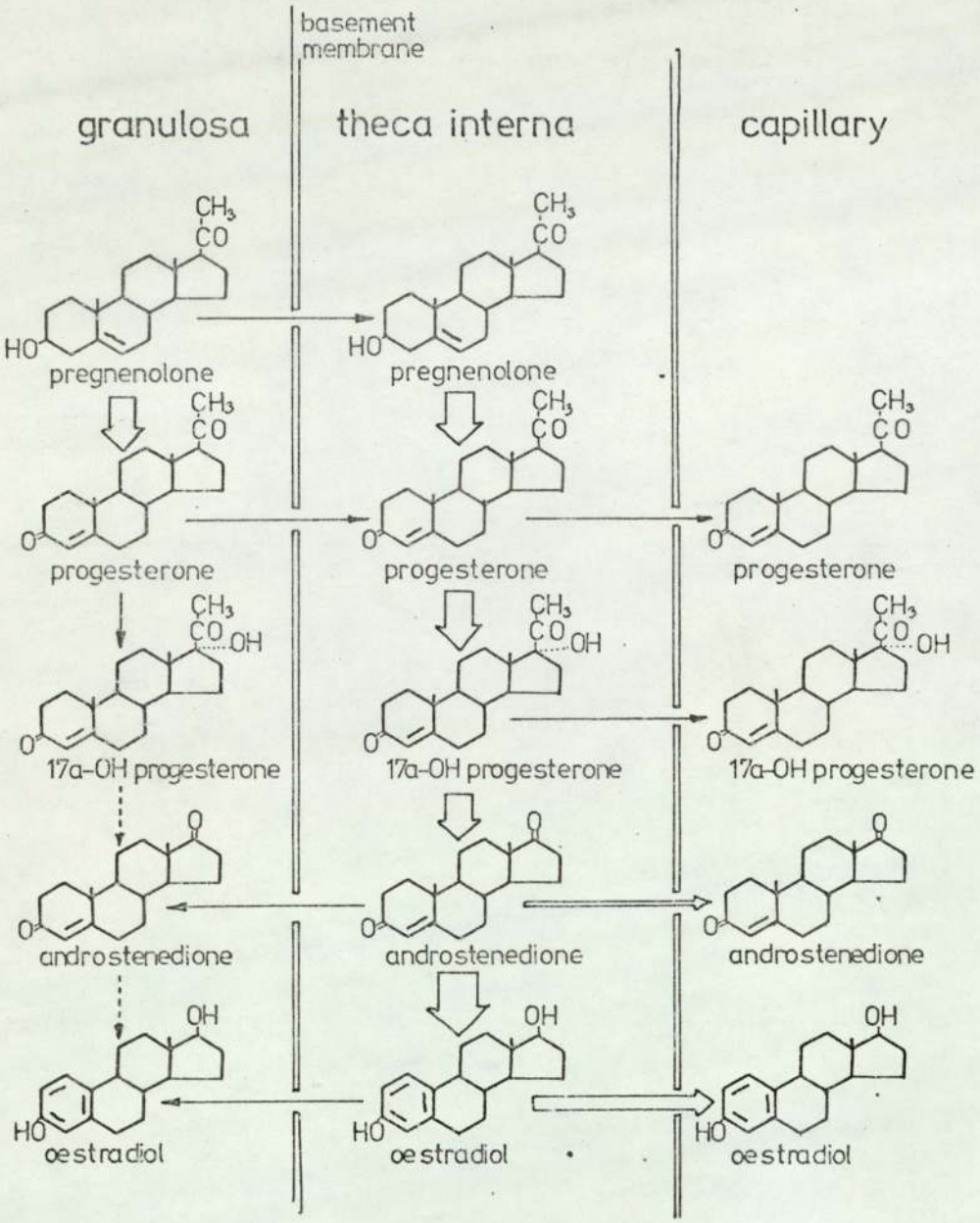



Fig.5:

Sites of oestrogen anabolism within the ovary. The thickness of the vertical lines is proportional to the degree of conversion or transport. (Adapted from Young[452])

or repressive inhibition at the transcriptional level) by the levels of free cholesterol. It used to be thought that bile acid levels could exert some control over hepatic cholesterol synthesis, although this idea is now disputed.

Thus, cholesterol provides a stable precursor pool for the subsequent oestrogen anabolism, and it is unlikely that oestrogen production is controlled to any dramatic extent by metabolic steps leading to the production of cholesterol. The subsequent stages, however, leading to the elaboration of the steroidal oestrogens, are very much implicated in the control of oestrogen levels. The control of the steroid production depends on the relative activities of the intracellular enzyme systems in the chain of steroid synthesis, these activities, in turn, being governed by the normal metabolic regulating factors such as substrate availability, feedback inhibition, adenylate control and active enzyme availablity. Thus, as Fig.5 shows, the theca interna cells are capable of synthesising oestrogens from precursors such as acetate or cholesterol, but the luteinising granulosa cells carry the synthesis only as far as hydroxy-progesterone. The cause of release of specific hormones into the circulation remains a mystery. Release cannot simply be due to accumulation beyond storage capacity; for in experiments in women with both ovarian veins cannulated the ovary containing the ripe follicle secreted about five times more

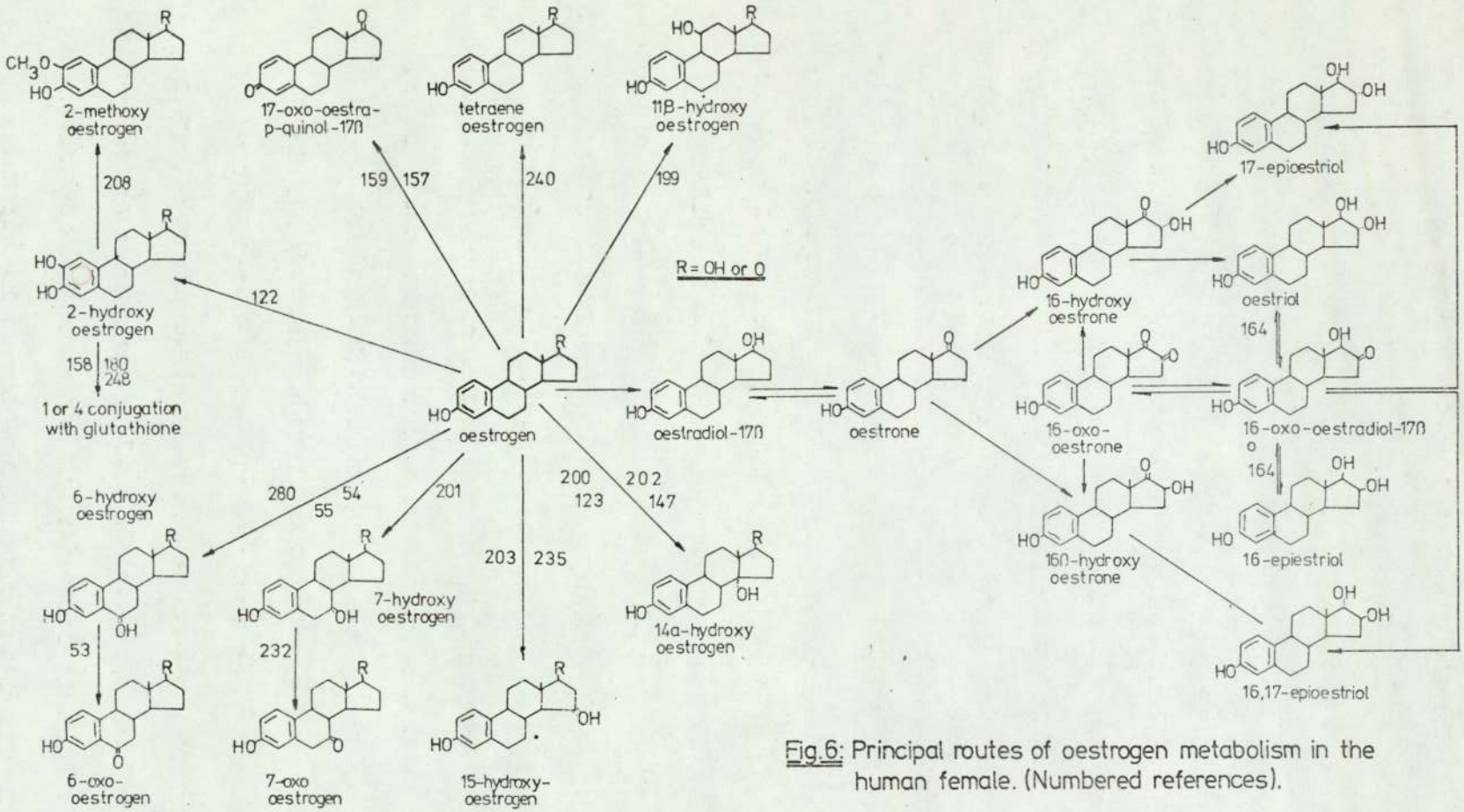



Fig.6: Principal routes of oestrogen metabolism in the human female. (Numbered references).

oestradiol than did the contralateral ovary, yet both ovaries secreted identical amounts of the major steroid precursor androstenedione. Clearly there are other factors in operation that may explain the release process. These probably include cellular permeability to the entry of substrate and exit of newly synthesised hormone(s), relative solubility and diffusibility of these compounds in interstitial fluid, and capillary permeability to the entry of the various products of synthesis.

The fate of the oestrogens once they have been secreted can lie in two directions. They may exert a biological effect, either directly or via a metabolite, or they may be excreted. (They do not appear to be stored in depôts to any great extent). The bulk of the steroidal oestrogens secreted by the adrenals and the ovary are excreted after a metabolic sequence which may be single step or may be tortuously complex. The interconversions, red-ox and conjugation reactions to which the oestrogens are prone are complex, and, in many aspects, are relatively little understood. Further comment will be made about some of these metabolic sequences in subsequent sections, so at this stage it is appropriate to establish those metabolic reactions which represent the principal routes of interconversion of the various oestrogenic steroids in the body fluids. The reactions known to occur in the human female are shown in Fig.6.

## IV Molecular Basis for Oestrogen Activity

I believe there to be at least three discreet levels of complexity and organisation within the cell at which oestrogens can exert control over the metabolic functions of the organism: the level of the cell, the level of the membrane of the cell, and at the level of the individual reactions within the cell.

The first model of oestrogen action implies an activity on the whole cell at the genetic level, and is the most widely researched and best understood aspect of steroid hormone activity. It seems that many of the aspects of this model, which have been established using oestrogen as the investigative tool, also have implications and relevance to the actions of many other steroid hormones, and it may well be that there is a common mode of action for this class of compounds.

The first concept to introduce is that of 'target' tissues. Target tissues are those which respond specifically to physiological levels of the steroid, and differ from non-target tissues in their ability to synthesise 'receptor' protein(s) which are responsible for the specific uptake of that steroid[173,300]. (Thus, an oestrogen target tissue is one which displays these properties with oestrogen). In these tissues, steroid penetrates the cell membrane and interacts with the receptor proteins present in the cell. After binding to the receptor,

the steroid-receptor complex is then transferred to the nucleus, a process which involves some alteration in the structure of the complex. Inside the nucleus, the complex binds to nuclear chromatin at specific acceptor sites, and this, in some way, allows ribonucleic acid polymerase (RNApol) to transcribe specific sections of the deoxyribonucleic acid (DNA) which would not otherwise have been read. Increased synthesis of ribosomal and messenger ribonucleic acid (rRNA & mRNA), with subsequent increased protein synthesis on the ribosomes, follows rapidly. It is appropriate, now, to look at some of these events in greater detail so as to present a lucid impression of the classical ideas of oestrogen action at the molecular level.

As has been previously stated, the oestrogen molecules (those molecules which possess oestrogen activity) are circulating in the blood of both men and women. The levels of these molecules are main-tained under control firstly by regulation of their rates of synthesis and release, and secondly through the activity of the SHBG servo (see Section VI ). (It is not thought that the conjugated oestrogens are free to play a dramatic biological rôle, and conjugation remains a mechanism of protection from excessive concentrations of hormone, rather than a fine concentration control system).

Thus we have the bulk of the oestrogens in the blood bound to protein, some unspecifically (to

albumin for example) and some with a relatively high degree of specificity (to SHBG). All this oestrogen is inactive, although it retains a potential for activity. The oestrogen which is free to exert a genetic effect is that which is freely circulating, unbound and unconjugated, and this represents a very small proportion of the total oestrogen complement of the blood. However, the bound and unbound fractions exist in equilibrium with each other, and hence the protein bound oestrogen is a large reservoir of potentially active hormone which can be mobilised by shifting the equilibrium position, or by depleting the free oestrogen fraction. The free fraction can be depleted by catabolic reactions, by conjugation, or by target tissue interaction (because oestrogen target tissues such as uterus and vagina possess the ability to concentrate and retain the hormone without chemical transformation[183]).

There is a great deal of controversy over many of the aspects of the hormone action, but there is general agreement that the initial stages of the humoral stimulus consists of a (passive) diffusion of free oestrogen through the plasma membrane of the cell. If the cell happens to be a target cell, then the oestrogen-binding protein (receptor) binds the incoming hormone in the cytoplasm, moves to the nucleus and interacts with the chromatin. These events are summarised in Fig.7. overleaf.

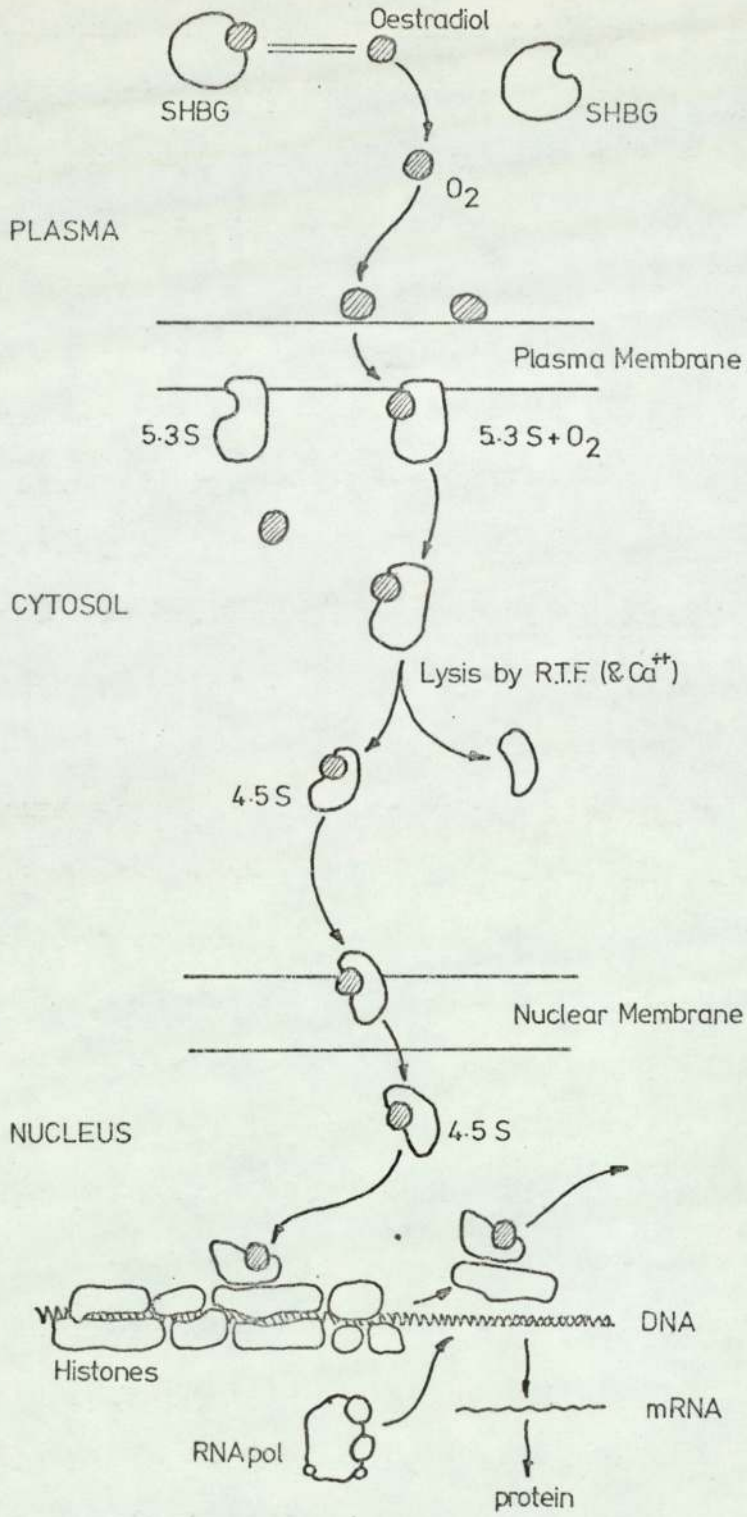



Fig.7:
Scheme to show the stages of oestrogen interaction with a target cell resulting in transcriptional control

There are thought to be three different forms of oestrogen-binding protein. They are characterised by their sedimentation coefficients, 'S'. (This coefficent is determined by centrifugation studies, and is a parameter of molecular shape and size; the smaller and more spherical molecules have a low S value). The three proteins are termed '8.6S', '5.3S' and '4.5S'; there is no indication that they differ in the nature of their oestrogen binding sites[323]. Both the 8.6S and 5.3S proteins can be extracted from target tissue cytosol, and the 5.3S protein can be prepared from the 8.6S by elevating the salt concen--tration of the extraction medium. The 8.6S to 5.3S conversion is reversible, and there is evidence to suggest that the 8.6S state results from the longi--tudinal, noncovalent association of two 5.3S molecules[51]. Considering that <u>in utero</u> the concentration of potassium plus sodium is close to 0.2M, the presence of the 8.6S state <u>in vivo</u> is doubtful, and the 8.6S form is probably an artefact of preparation caused by the use of low salt buffers[51].

The 4.5S molecule is a different, smaller oestrogen-binding protein which is obtained from cell cytosol when calcium is present in high salt medium (KCl greater than 0.1M). It is stable, even in low salt buffers, and is much less asymmetric than the 5.3S protein; in addition, its formation appears to be irreversible. It appears that the 4.5S protein is formed from the 5.3S protein by the action

of a proteolytic enzyme which is calcium dependent. This protein has been called the 'receptor trans--forming factor' (RTF), and the evidence suggests that the 5.3S is cleaved into two, approximately equimolecular, fragments by the action of this RTF[51,449]. This 4.5S fragment has been extracted from the cyytosol of target tissue cells, and also from the nucleus; the nuclear protein being indistinguishable from the cytoplasmic one not only in sedimentation rate, but also in Stokes radius, molecular weight, electro--focusing position and all other properties studied. The 4.5S protein can be extracted with oestrogen bound to it, and currently it is believed that the oestrogen enters the cytoplasm and forms a complex with the 5.3S receptor protein (which is confined in the cytoplasm, possibly by being bound to the inside face of the plasma membrane, or to some other cytoplasmic structure). The calcium-activated RTF then acts on the 5.3S-oestradiol complex, splitting off a 4.5S oestradiol-binding fragment, and this derivative, mobile complex penetrates the nucleus where it interacts, strongly though reversibly, with the chromatin.[449]

Preliminary characterisation of the nuclear binding sites has shown that they are probably proteins, and that they are basic in character. They are able to bind only oestrogen-receptor complexes, and not the free 4.5S protein. There are very large numbers of these binding sites (typically $10^4$-$10^5$ per

nucleus in uterine cells[181], and their affinity for oestrogen-receptor complexes is high ($K_{dis}$ less than $10^{-9}$ M at 4°). That there are a large number of active nuclear binding sites is supported by the observation of a large burst of RNA synthesis in responsive cells after administration of oestrogen, consisting mostly of rRNA and transfer RNA (tRNA), but also including mRNA; as well as the large increase in template capacity of chromatin (up to 50%) within one hour from oestrogen administration[133], which is quite in line with activation of a large proportion of the genome.

The oestradiol-receptor complex has been found to bind to DNA prepared from uterus[195], spleen[408], liver[74], and thymus[449], as well as that from Escherichia coli and Bacillus subtilis[408,74]. At present there are no compelling reasons to accept or reject the possibility of these DNA acceptor sites being of physiological relevance. In addition, it is known that the uterine oestradiol-receptor complex will bind to isolated chromatin prepared from uterus[395], mammary tumors[259], and spleen, liver, lung and kidney[395]. The question of whether isolated uterine chromatin displays a specificity for the receptor complex which is not shown by non-target tissue chromatin has been approached by studying the binding kinetics of the reaction, and this question of a tissue-level specificity in the binding of the hormone-receptor complex to isolated chromatins (and its possible

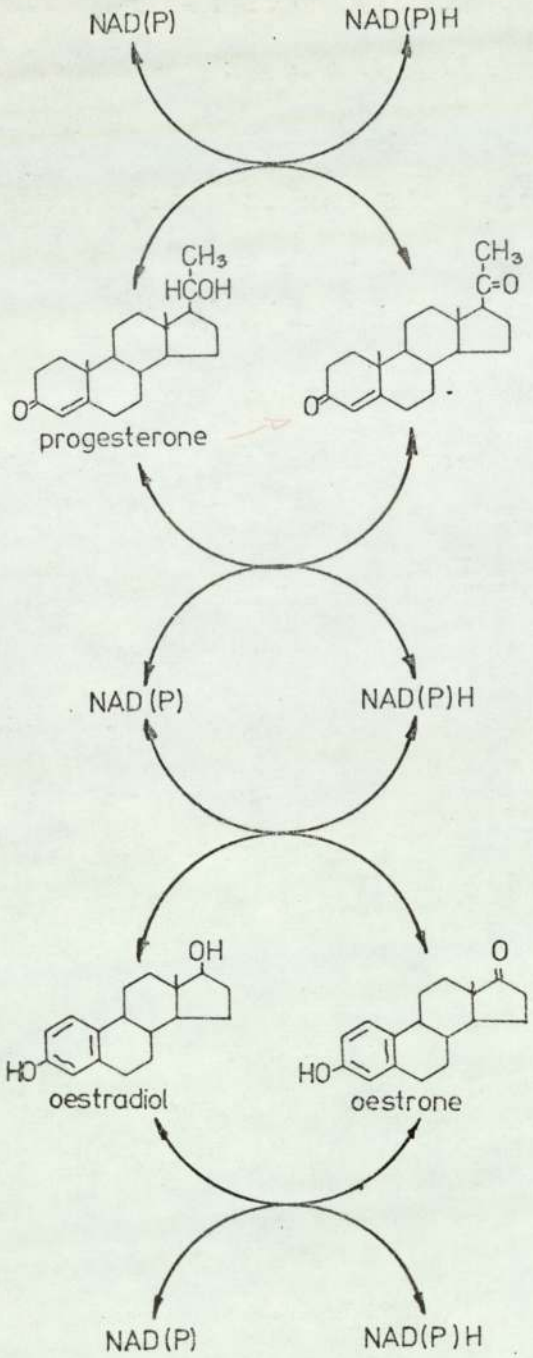



Fig.8:

Showing the possibilities which exist for oestrogens (and androgens) to be involved in metabolic red-ox reactions of cellular intermediary metabolism.

in vivo consequences) are still a matter for debate. The topic has been well reviewed recently[182,443], and is the subject of much current research. The scheme shown in Fig.7, although capable of being challenged in detail, represents the accepted mode of oestrogen interaction with target cells as understood at the present. The general principles it implies, in particular the transcriptional basis of oestrogenic control[263] have been confirmed in many experiments with a variety of model systems.

As mentioned earlier in this section, there is more than one level at which oestrogen can exert a biological activity in vivo, and much debate surrounds, not the accuracy of observed effects, but their relative importance to the cell. So, while transcrip--tional control exerted by oestrogen may be undisputed, there still exists the possibility that this is not the principal axis of oestrogenic control of cellular function. In this thesis I shall try to be dispassionate about these different systems, and not labour their relevance.

Whilst oestrogens can act at a genetic level, needing the full cellular organisation in order to do so, they can also act at metabolic stages of cellular processes, and these effects do not require a concerted cellular behaviour in order for them to be apparent. Some need only intact organelles, and others can be demonstrated in cell-free systems with only one

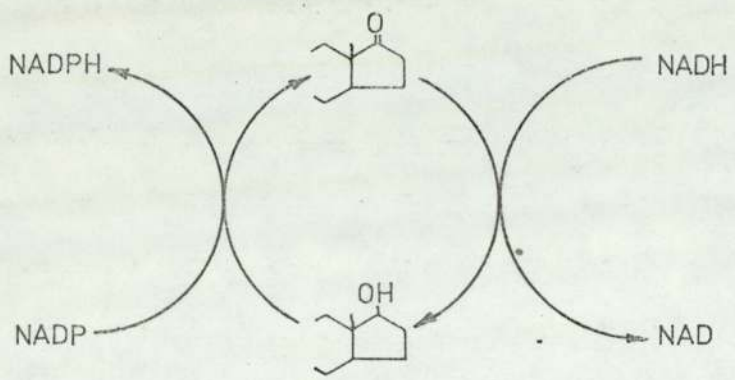



Fig.9:
Possible involvement of oestrogens in the transfer of reducing power from the mitochondria to the cytoplasm.

known reaction occuring. For example it has been reported that (relatively high) concentrations of oestradiol disrupt the tetrameric quaternary structure of the key metabolic enzyme glutamate dehydrogenase[246]. Thus there is a possibility that oestradiol exerts a regulatory effect on certain control centres of the metabolic pathways of the cell by direct activation-deactivation reactions. Similarly, it has been suggested that steroids such as oestradiol might act as coenzymes for some enzymic reactions. Since physiological concentrations of oestradiol have been shown to stimulate NAD-dependent isocitrate dehydrogenase activity in placental tissue[102], and Pollow has suggested[318] that the hydrogen transfer between steroid hormones represents an aspect of the true reaction mechanism of steroid hormones (Fig.8). The results of several experiments suggest that steroid hormones function as hydrogen-transferring coenzymes by forming part of a chain of hydrogen carriers, as, for example, in the transfer of reducing power between the cytoplasmic fraction and the mitochondrial inner space, where NADH acts as the starter substance for the respiratory chain, but is unable to be transfered to the cytoplasm because of the impermeability of the mitochondrial inner membrane (Fig.9). These effects of oestrogen are exerted at the molecular level on individual reactions. The activity of oestrogen at the membrane level, however, is very different, and will be discussed later.

## V Oestrogens in Pregnancy

Perhaps the most unique feature of steroid metabolism during pregnancy is the precursor role of circulating steroids for the production of hormones such as oestrogens by the placenta. Pregnancy characteristically involves the processes of placentation, interuterine development, viviparity and postpartum lactation, with all these processes depending, very largely, on the specific actions of oestrogenic and progesta--tional hormones. These steroids are synthesised during gestation by the maternal ovaries or fetal placenta, or both, in concert.

Although pregnancy begins for the fetus at the time of fertilisation, it begins for the mother only when the presence of the blastocyst is recognised by the maternal organism and the ovarian cyclicity of the menstrual cycle is replaced by pregnancy-specific endocrine mechanisms. The cues to the mother initiated by the blastocyst may be altered uterine metabolism and/or secretion of gonadotrophin, which independently or in concert transform the corpus luteum of the cycle into the corpus luteum of pregnancy, with a lengthened life span and augmented steroid production[419].

It appears that during the course of pregnancy the placenta assumes increasing degrees of steroid producing activity and eventually replaces ovarian

function completely[13].

At one time, the fetus was considered to be a non-participant in the endocrinology of pregnancy but it has now been implicated, along with the placenta, in the active biosynthesis and metabolism of steroid hormones[100]. In addition, the maternal (and fetal) adrenal glands appear to have reproductive, as well as homeostatic, functions in the endocrine control of pregnancy[23]. However, much of the classic literature on endocrine function during pregnancy is often simply descriptive. Measurement of steroid hormones has often been by relatively non-specific bio-assays or chemical assays too insensitive for the low levels of hormones found in some body fluids and tissues[286,350]. The more recent application of radioimmunoassay, competitive protein binding, and refined chemical techniques for assay of steroids, together with the introduction of experimental fetal surgery and extension of detailed placental biochemistry studies have begun to reveal the complex interactions which exist between mother, placenta and fetus; in particular the essential and active role of the fetus in the metabolism of oestrogens.

The concept of the fetus and the placenta as a functional endocrine unit - the feto-placental unit, FPU[98] - responsible for most of the oestrogen production during pregnancy has led clinicians to use the oestrogens present in maternal urine or

plasma as indeces of the fetoplacental function.

It is generally accepted that the biosynthesis of the three classical oestrogens (oestrone, oestadiol-17B and oestriol) follow somewhat different metabolic pathways. This has been briefly described in Fig 2, which shows that the main precursor of oestrone and oesradiol-17B is dehydroepiandrosterone (DHA). This steroid is delivered to the placenta mainly in a conjugated form as dehydroepiandrosterone sulphate (DHAS)[215]. Most of the DHA derives from the fetal adrenals, but the maternal contribution is probably substantial[380].

The metabolic pathways for oestriol formation are somewhat different. The placenta lacks the 16a-hydroxylating enzymes which are necessary for the conversion of DHA to oestriol. Instead, large amounts of DHA produced by the fetal adrenals are hydroxylated by the fetal liver to 16a-hydroxy DHA[41] which is converted to oestriol by the placenta[93]. Other pathways have also been described[94,333,427]. Although the mother probably contributes considerable amounts of precursors for oestrone and oestradiol-17B synthesis in the placenta her contribution of precursors for the oestriol production is thought to be minimal[379,380].

Oestriol is quantitatively the dominant oestrogen during pregnancy. Its production, unlike oestrone and oestradiol-17B, is largely confined to the FPU. It is thus not surprising that

determinations of the maternal urinary excretion of oestriol or total oestrogens have long been used in attempts to assess the fetoplacental function in abnormal pregnancies.

The normal values for oestrogen concentrations of pregnancy plasma at differing stages of gestation have been determined by very many workers. The results obtained have been well reviewed by Jayle et al.[176], and more recently by Lindberg et al[229].

There is little point in reproducing their criticisms, except to suggest that, in the case of the plasma assays, the more recent determinations (and hence those dealt with exclusively in the later review) are more likely to be more trustworthy values. Even when a method has been used which has a high degree of accuracy and sensitivity, the results are still subject to some variations as a result of uncontrolled (or unknown) factors in many cases. For example, the magnitudes of the endogenous fluctuations in the plasma levels of the oestrogens have an important bearing on the application of plasma analyses of these hormones in clinical practice. The day to day variation of the total plasma oestriol levels was studied by Masson and Wilson[252] who found a coefficient of variation of 27%. Other workers[229] found that levels of unconjugated oesrogens may show large and rapid fluctuations. With even week to week changes of the relative levels of the unconjugated fractions of all three oestrogens showing coefficients of variations in excess of 30%.

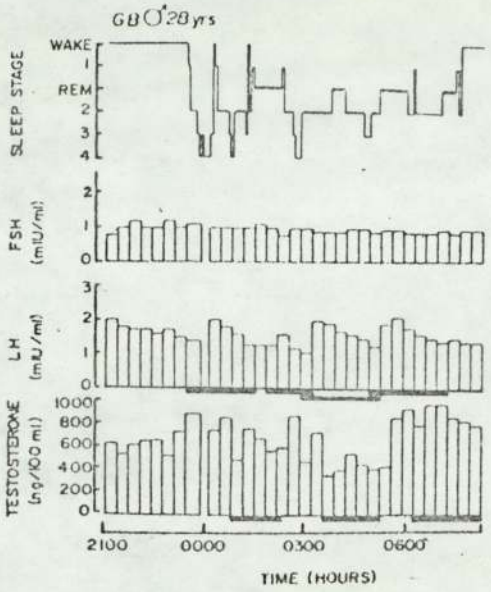
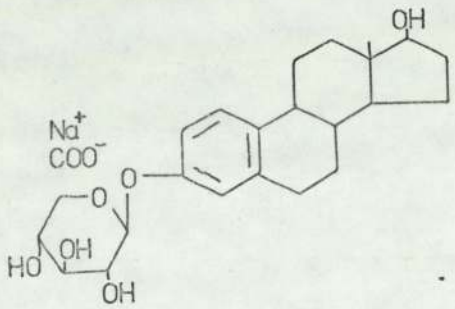



Fig.10:

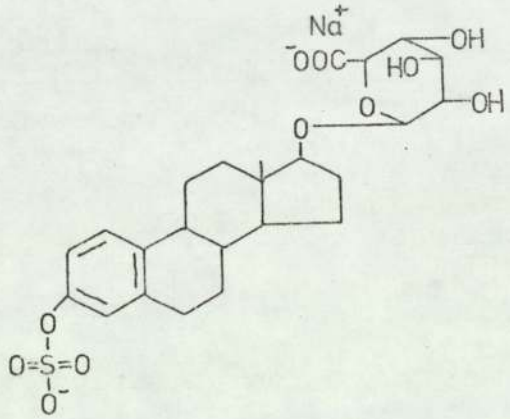
Integrated concentrations of LH, FSH and oestradiol and stage of sleep in women. The significant short-term fluctuations are underlined. These were defined as abrupt or progressive rises in hormone level followed by progressive declines over at least two consecutive samples where the peak level was significantly different from both the preceding and following lowest levels. (From Alford[10]).

The diurnal variations of the plasma levels of oestrone, oestradiol and oestriol have been investigated by different authors with conflicting results. In four studies of the levels of the total oestriol in plasma no significant or systematic fluctuations during a 24 hour period could be demonstrated[191,245,252,348]. One group, however, found significant fluctuations in total oestriol with a diurnal rhythm characterised by lower levels at 4.30pm than at 8.00 or 9.00pm[371]. It has been noted[409] that the individual daytime means of unconjugated oestradiol were significantly larger than those recorded at night time. Fig.10 shows data from Alford et al.[10] showing a significant temporal pattern of integrated plasma oestradiol levels during sleep. The circadian rhythm of total oestriol was less marked than for unconjugated oestradiol, and marked fluctuations in the plasma levels of unconjugated oestradiol have been found in samples of hospital patients[281], the levels at 10.00pm being lower than at 8.00 am and 4.00 pm.

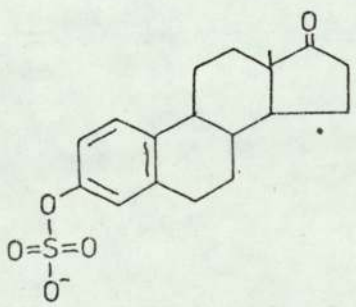
The results of a more recent report[229] indicate that the endogenous fluctuations in total oestrone and oestriol are fairly small, while significant variations in the levels of unconjugated oestrone and oestriol are common. This might be explained by the assumption that the release of these hormones from the placenta if fairly constant while the degree of conjugation undergoes rapid changes, however this




ß-Oestradiol-3-ß-glucuronide




ß-Oestradiol-3ß-sulphate-17ß-D-glucuronide




Oestrone-3-sulphate

Fig.11:

Typical conjugated oestrogens present in pregnancy urines.

is speculation, and no recent results support the idea except for those of Townsley et al.[409].

Urine

As with the plasma oestrogens, urinary oestrogens have attracted interest for a considerable period of time. However, because of the high concentrations associated with pregnancy urine, particularly in the later stages, the earlier work in this field is much more trustworthy than was the early work on plasma oestrogens. The topic is exhaustively reviewed in Jayle et al.[176] who notes that although about 15 phenolsteroids have been isolated from the urine of human pregnancy (in fact most of the molecules in Fig.6) most are secreted in small quantities, and the bulk of the total urinary oestrogens is made up of the three classical oestrogens (oestrone, oestradiol and oestriol) together with the ketolic phenolsteroids (16a-hydroxyoestrone and 16-keto oestradiol). Most of the determinations of urinary oestrogens which have been made, at some stage, involved an extraction with sodium carbonate solution, and since the ketolic phenolsteroids are destroyed in this environment, it is highly likely that in practice most of the data obtained to date concerns only the three classical oestrogens. In urine the greater part(99.5%) of the oestrogens is conjugated, 95% with glucuronic acid, and 4.5% with sulphuric acid[177] the structure of typical conjugates

| Weeks | Mean µg-24 hr | Extreme values |
|---|---|---|
| 5-6 | 150 | 85- 300 |
| 7-8 | 245 | 135- 490 |
| 9-10 | 345 | 190- 690 |
| 11-12 | 500 | 275- 1,000 |
| 13-14 | 865 | 475- 1,730 |
| 15 | 1,500 | 600- 2,000 |
| 16 | 2,200 | 1,050- 3,000 |
| 17-18 | 3,200 | 1,475- 4,800 |
| 19-20 | 4,600 | 2,500- 6,500 |
| 21-22 | 6,000 | 3,000- 7,500 |
| 23-24 | 7,400 | 3,500-10,500 |
| 25-26 | 8,400 | 4,000-11,500 |
| 27-28 | 9,300 | 4,500-12,500 |
| 29-30 | 9,600 | 5,000-14,000 |
| 31-32 | 10,800 | 6,000-15,000 |
| 33-34 | 16,000 | 9,100-24,700 |
| 35-36 | 19,000 | 10,400-27,300 |
| 37-38 | 23,500 | 12,420-37,260 |
| 39-40 | 25,000 | 14,200-41,180 |

Table 1:

Excretion of total oestrogens during normal pregnancy. (From Jayle[176]).

is shown in Fig.11. The percentage of ester sulphates increases towards the end of pregnancy and they may ultimately constitute 20% of the conjugated oestrogens[177]. The distribution of oestrogens does not differ appreciably from one fraction to another when free, the glucos--iduronate and the ester sulphate fractions are compared. In all three fractions, about 20% is oestrone, 5% oestradiol and 75% oestriol[177]. The extent and complexity of conjugation is great, with a wide variety of different oestrogen conjugates having been discovered[24,25,66,155,243], nevertheless, the 16a-glucosid--uronate is the most important conjugate of oestriol, and hence the most important conjugated oestrogen[66,155].

As mentioned earlier, the conjugates of oestrogens, particularly the glucosiduronates, are cleared very rapidly from the blood, and it is clear that this clearance, and the subsequent excretion into the urine, represents the greater fraction of the oestrogens synthesised in the body. As a consequence, the estimation of oestrogens in 24 hour urines provides an index of the total quantities of oestrogens elaborated by the ovaries and placenta, particularly during the course of pregnancy, but naturally does not give much information about the short-term fluctuations in the rates of synthesis. Table 1 shows the excretion of total oestrogens at different stages of pregnancy, and Fig.12 shows the profile of the change in the excretion of these oestrogens. The general form of the curve is that of a double sigmoid. Right from the

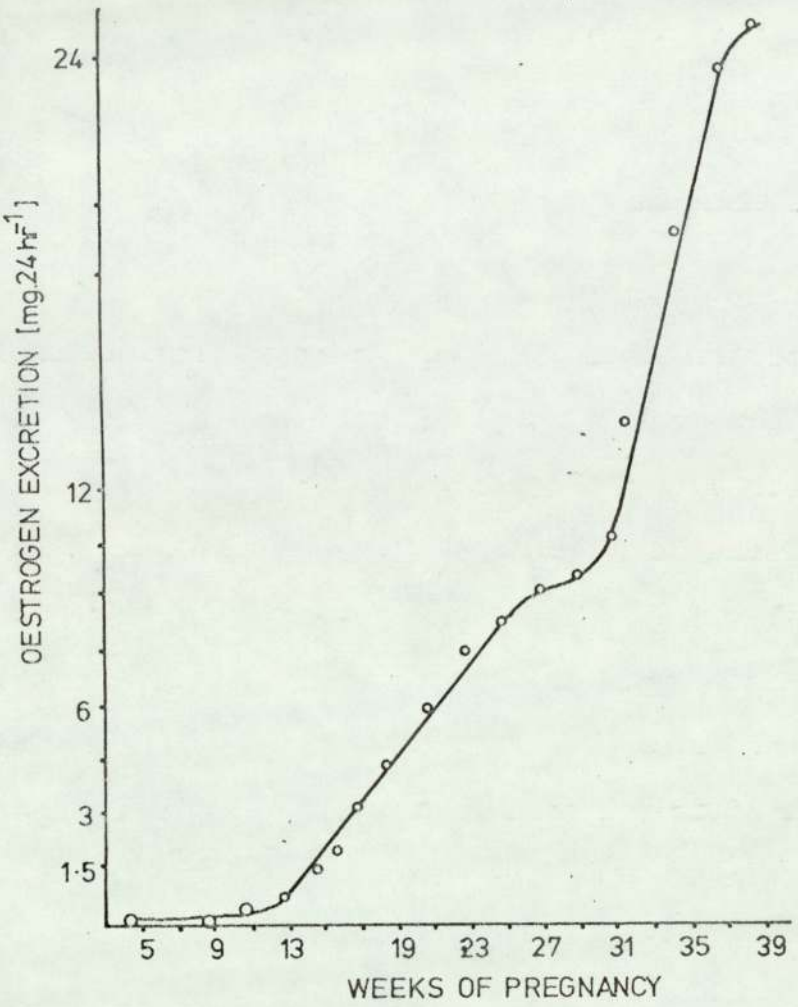



Fig.12:

Urinary excretion of oestrogens during pregnancy showing the mean normal curve from a sample of 1,560 pataients with uncomplicated pregnancies. (Adapted from Jayle[176] ).

start of pregnancy the quantity of urinary oestrogens exceeds that found during the luteal phase of the menstrual cycle, as shown in Fig.14, and there appears to be an exponential rise in excretion from the 5th. to the 32nd week, followed by a straightening of the curve, the slope of which is very steep during the last two weeks of pregnancy.

Amniotic Fluid

The fetus is contained within a membranous bag called the chorion, and this bag is distended with a fluid which thus bathes the conceptus. This fluid (amniotic fluid or liquor) has been described as fetal bath-water, but it is unlikely that this description is accurate, since the fluid appears to be in a state of dynamic equilibrium, rather than existing as a stagnant pool. It is known that oestriol concentration in the liquor increases throughout pregnancy, particularly after 34 weeks gestation[265]. In late pregnancy oestriol enters the amniotic cavity, largely as a result of fetal micturation[35,227]. Some of the oestriol is transfered to the maternal circulation by crossing the amnion and chorion[134], but there appears to be very little movement in the opposite direction. However, much of the hormone passes out of the amniotic cavity when it is swallowed by the fetus (up to 500 ml per day in late pregnancy[322,341]) subsequently returning to the liquor by fetal micturation or passing to the placenta, and then on to the maternal circulation.

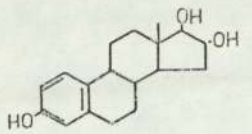
Many workers have studied levels of oestrogens in amniotic fluid during pregnancy, and there is a recent review[197] dealing with this topic. There appears to be general agreement about the mean levels of oestrogens in normal subjects, and also about the considerable range of the normal results. Representative figures[116] show a mean and standard deviation of 125 to 36 ug per 100 ml in late pregnancy. There is a very wide scatter of normal values, though, which may reflect the dynamic equilibrium existing between the maternal and fetal compartments.

## VI Oestrogen Levels in Body Fluids

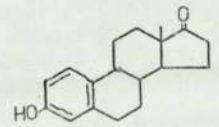
All of the oestrogen metabolites described in Fig.6 have been identified in the blood or urine of women. Since there is no clear evidence of any extensive modification of oestrogens by the kidney, it is probably fair to assume that if a metabolite is present in the urine of a subject, then it is also likely to be present in the plasma, albeit at a different concentration.

In general, the concentration of oestrogens in the blood/plasma is considerably less than that in the urine, and this has presented considerable difficulties to workers attempting to assay the low levels of oestrogens found therein. Comparison of the values for oestrogen concentrations of peripheral human blood is difficult because many different methods have been tried, and, in the past, many of the methods used were operating at their lower levels of sensitivity. In some cases, the values presented are at great variance with those offered by more recent workers. I feel that, in this case, the more recent results are likely to be the most trustworthy, and to reflect, most accurately, the true pattern of oestrogen concentrations in the plasma. The methods which have been used to determine oestrogens in plasma (and also in urine, bile and other tissues) will be reviewed in the next sections. At this stage it is appropriate to record the normal values for

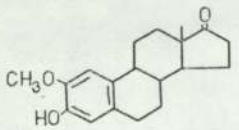
Fig. 13



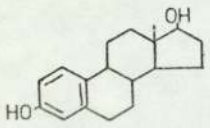

oestriol



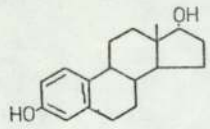

oestrone



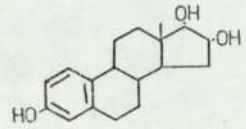

2-methoxyoestrone



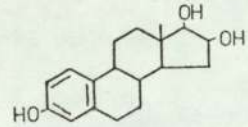

oestradiol-17β



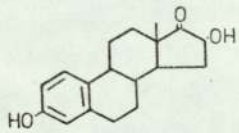

oestradiol-17α



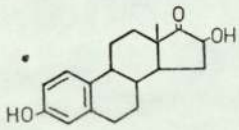

17-epioestriol



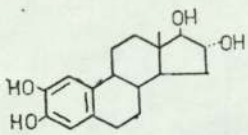

16-epioestriol



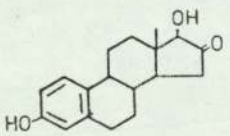

16α-hydroxyoestrone



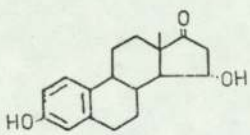

16β-hydroxyoestrone




oestetrol




16-oxo-oestradiol-17β




15α-hydroxyoestrone

the oestrogen concentrations of tissues of non-pregnant women as determined by the most recent methods. Fig.13 shows those oestrogens which have been positively identified in plasma. Most of these compounds are present in trace quantities only, and probably do not exert any particularly profound biological effects. In any case, their determination is often extremely difficult, and the results are frequently open to question. Fig.14 shows the normal range of concentrations of the two classical oestrogens to be found in the peripheral plasma:

| Source | 01 | 02 |
|---|---|---|
| Follicular Phase | 60 | 30-100 |
| Luteal Phase | 110 | 130-470 |
| Male Plasma | 60 | 20-60 |
| 15/52 Pregnant | 1,000 | 4,000 |

Fig.14: Concentrations of free oestrogens in peripheral plasma ($pg.ml^{-1}$). Data from references 7 & 301. (01: oestrone, 02: oestradiol)

These values are for the concentrations of free (i.e. unconjugated oestrogens. Very few workers have been altruistic enough to study the levels of conjugated oestrogens in plasma, since it has been assumed that only the free forms are capable of exerting any biological effect, and most studies have been conducted in an atmosphere of clinical relevance. In plasma, the compounds shown in Fig.13 will all

be present as conjugates (mainly sulphates) with very little material present in the free form (except in the case of oestradiol). There appears to be an equilibrium between the sulphated, the free and the glucuronidated forms, with the latter being cleared very rapidly by the kidney, leaving the sulphates as the predominant forms. The body is undoubtably responsive to the levels of the free (active) oestrogens, and we have developed subtle control systems to main--tain these levels within certain limits. The conjug--ation reactions resulting in the glucuronidated oestrogens are part of these systems, and apart from conditions of endocrine disorder, the circulating levels of free oestrogens in the peripheral plasma are in fact maintained within accurate limits. There are diurnal variations in the levels[335], and it has been noted that severe dietary (or perhaps emotional) stress may affect the circulating levels, as in the case of anorexia nervosa[32] . The blood contains several buffer mechanisms for guarding against sudden changes in concentration of oestrogens which might occur because of a change in the rate of production, or even because of the effect of bodily position on their metabolic clearance rate (MCR). The MCR varies according to hepatic blood flow[400] and hepatic blood flow is greatest when the patient is in the supine position. In fact, there is a 35% fall in the liver blood flow when a supine position is exchanged for an erect one[231]. The relative importance of these buffers has yet to

be established. Some are obviously uncontrollable, and apply to equilibrium parameters, such as the buffer stock of sulphated oestrogen which is in equilibrium with the much smaller stock of (active) free molecules. Others are likely to be under more control on a long term basis, like the buffer stock of oestrogen bound to the external surfaces of blood cells such as the erythrocytes and the blood platelets.[313] In the female peripheral blood about 10% of both free oestrone and free oestradiol is associated with the red cells[236,429], and both oestradiol and oestrone are known to be bound to platelets, the platelets being thought to effect conversion of a proportion of oestradiol to oestrone[314]. The most sensitive, and perhaps the most important buffer is probably the stock of unconjugated oestrogen bound to the plasma proteins, in particular that bound to SHBG. Oestrogen binding to albumin is probably not controllable to any great extent, but that bound to the SHBG is subject to a very sensitive 'servo' mechanism for regulating the relative levels of free oestrogen and testosterone[15].

SHBG binds testosterone with more avidity than it does oestrogen. When bound, the steroids are very much less sensitive to degradative attack, and general hepatic metabolic clearance[237,353], but there is considerable evidence that it is only the unbound fraction of the free oestrogens that is biologically active[279,330]. Now, SHBG binds testosterone more readily

than it binds oestrogen; but it has also been established that administration of androgens (in both sexes) causes a reduction in SHBG level, and that oestrogens cause a rise[282,407]. In fact the level of SHBG appears to be remarkably sensitive to a change in relative production of androgens and oest--rogens; (the administration of ethinyl oestradiol, at a rate of 20 ug per day,.to male criminal sex offenders produced a 150% increase in SHBG levels[15] ). These observations led to the concept of SHBG acting as an amplifier or servo for oestrogen/androgen balance[15] . If oestrogen production increases, the unbound oestradiol rises in concentration, and SHBG production is stimulated. Since SHBG binds testosterone better than it does oestradiol, the effect of this will be to reduce the levels of free testosterone and cause a differential fall in the rate of test--osterone to oestradiol, tipping the sex hormone balance further in favour of oestrogen. Conversely, a rise in testosterone production will amplify its own effect by causing a fall in SHBG and a differential rise in unbound testosterone relative to oestradiol. Furthermore, any other factor altering SHBG concent--ration may tip the balance in favour of testosterone or oestradiol, depending on the direction of the change in SHBG concentration. Thus, SHBG may form the basis of the most responsive and subtle mechanism by which the body controls the concentration of free oestrogen and androgen in the blood bathing its tissues.

For this reason, the clinical significance of the concentrations of circulating oestrogens, as detected by most methods, may not be all that great, and there is need for caution when assessing the significance of such data. The tissues of the body that have been categorised as 'target'tissues for oestradiol are all responsive to very small concentrations of the hormone, and in a later section of this thesis I will describe how other tissues of the body - not previously regarded as oestrogen-sensitive - might well prove to be capable of modification by small amounts of free oestrogen. We should really be trying to study the subtle and small changes in free oestrogen level to which these tissues are subjected, rather than studying the total blood content of unconjugated oestrogen. In much of the literature in this area the term 'free' in the context of oestrogens has been used as if it were synonymous with 'unconjugated' which is misleading and awkward. It would be better to reserve the term 'free' for use only with that oestrogen which is truly unrelated to either a protein binding agent or a conjugated acid moiety, which is probably the only active oestrogen in the blood.

With urinary oestrogen concentrations, the situation is rather different. Urinary values represent the amount of oestrogen excreted from the body, and therefore cleared from the blood, in a

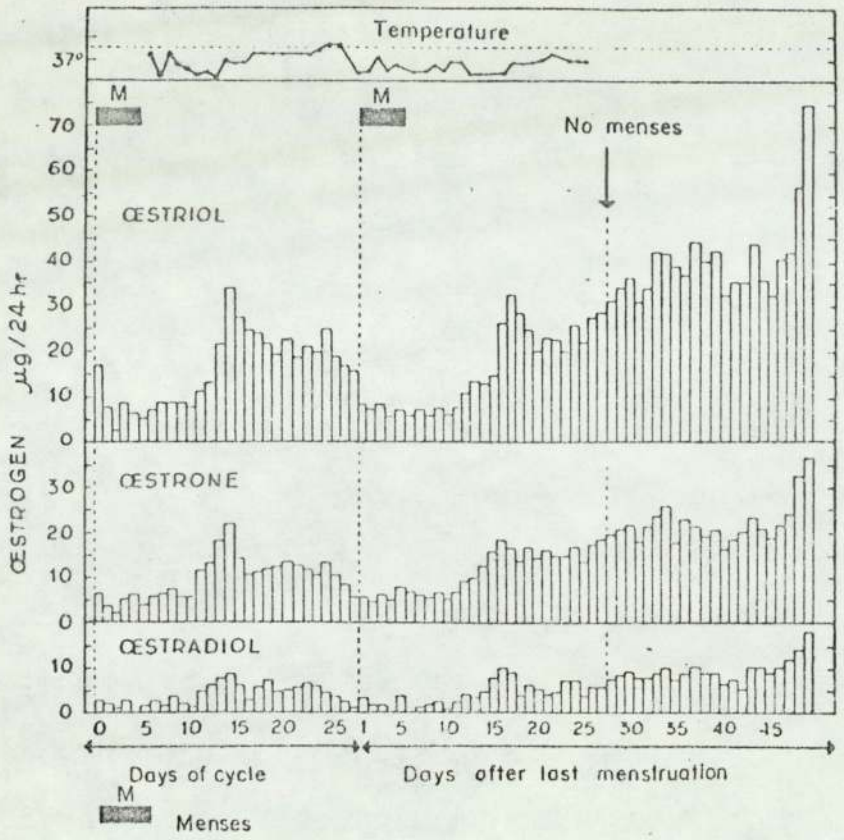



Fig.15:

Urinary excretion of oestrogens during a complete menstrual cycle and during a cycle followed by pregnancy. (From Brown[59] ).

given period of time (usually 24 hours), and are thus not subject to diurnal variation, although they are still subject to changes in bodily position (supine/erect) in the same way as plasma levels because of the changes in the renal blood flow. Clearly, when studying the urinary levels of oestrogens there is no attempt to evaluate the amounts of biologically active oestrogen to which the tissues of the body have been exposed. One is rather looking at the gross result of the bodily oestrogen metabolism over a considerable period of time. The information can be very revealing and valuable, as will be seen in the next section, and it is my opinion that urinary oestrogen levels should be complemented by plasma levels, rather than replaced by them as parameters of endocrine function in the pregnent and non-preg--nant subject. Fig.15 shows the pattern of excretion of oestrogens during the menstrual cycle. Oestriol is the predominant oestrogen, but all three major oestrogens follow the same pattern of excretion throughout the cycle. The majority of the oestrogen voided in the urine is excreted in the form of glucuronides, with very little sulphated steroid, and virtually none free. (Naturally, there should be none excreted as a complex with protein).

## VII Oestrogens in Complicated Pregnancy

In the preceding section I have noted the levels of urinary and plasma oestrogens associated with the different stages of pregnancy. All these values have been determined in subjects who were undergoing a normal, uncomplicated pregnancy. Since the importance of the feto-placental unit in the production of oestrogens has been recognised, it has become clear that a diseased, or otherwise unhealthy FPU, is likely to be less efficient in its oestrogen production than a normal one; and as a consequence, a decreased plasma or urinary oestrogen level will be symptomatic of an unhealthy FPU. As long ago as 1933, Spielman tried to correlate low urinary oestrogens with fetal death in utero[394] and found a significant relationship between the two. Since then, the value of (urinary) oestrogens has increased as clinicians discovered that many of the causes of fetal distress could be monitored using these oestrogen assays. It is not appropriate for me to review the (very copious) clinical data which is available on this topic, but it will set the scene for following sections if I briefly describe some of the complications of pregnancy which have been associated with low oestrogen excretion rates, and to indicate the value of oestrogen assays in these cases.

The first problem, however, is to establish

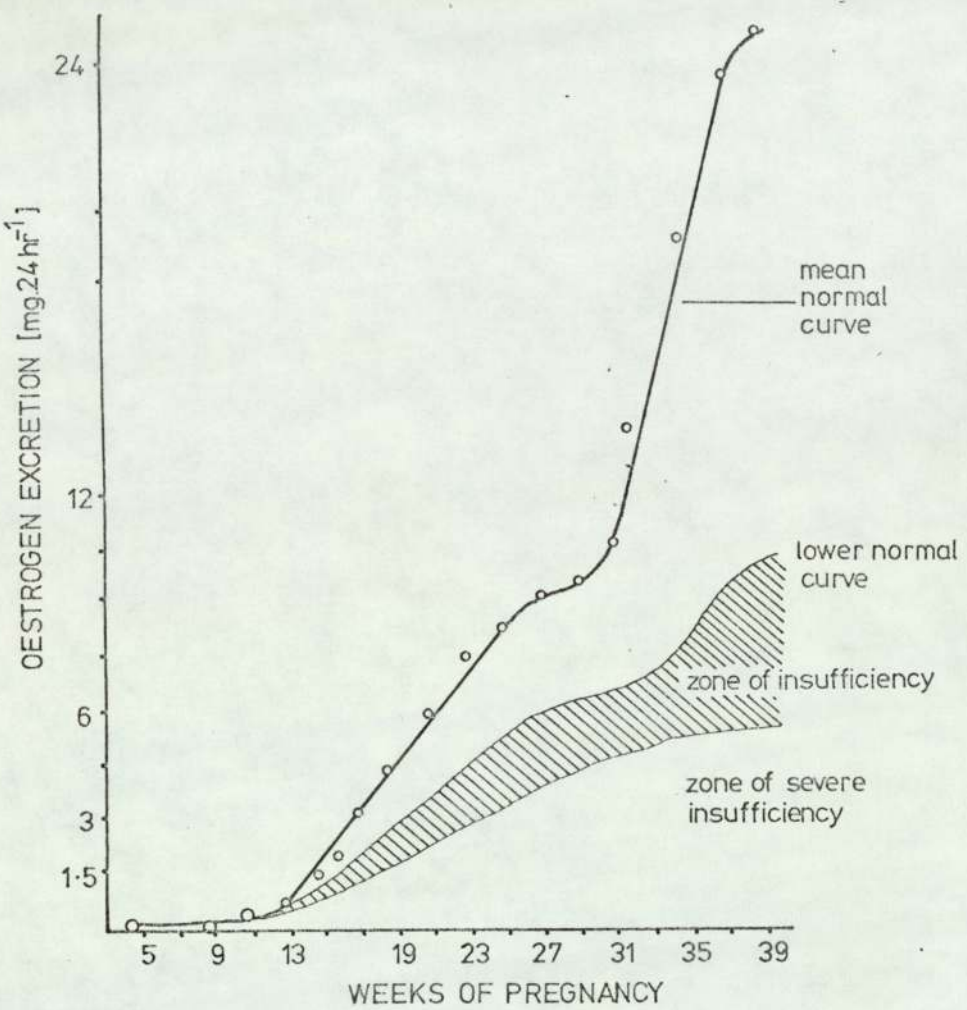



Fig.16:

Urinary excretion of oestrogens during pregnancy, showing the lower normal limit and the zones of feto-placental insufficiency which would give rise to alarm. (Adapted from Jayle[178] ).

'normal' limits for the oestrogen concentrations associated with 'normal' pregnancies. For reasons which have been discussed, urinary oesrogen determinations have been studied for many years and a great deal of trustworthy data has been accumulated which has enabled a clear picture of the normal range to be drawn. Fig.16 is taken from Jayle et al.[178] and shows the normal ranges calculated from several thousand determinations of urinary oestrogen concentration. Plasma levels are less well documented, and in general have not been used extensively as parameters of fetal wellbeing. However, it is a fair generalisation that, although not all complications of pregnancy result in decreased oestrogen production and excretion, lower than average oestrogen excretion nearly always indicates a clinical problem. Fig.17 shows the normal distribution of plasma oestriol (unconjugated) during the course of pregnancy, as determined by radioimmunoassay[73], and it is clear from both these figures, that there is a very considerable range of normal values. When a complication of pregnancy is suspected, it is usually detected because the trend of oestrogen concentration is abnormal. That is, if the urinary oestrogen concentration suddenly starts to decrease after the 32nd week, instead of continuing to rise sharply, then that would be a good indication that all was not well; whereas, a patient might show a very low oestrogen value at the 32nd week, but still be

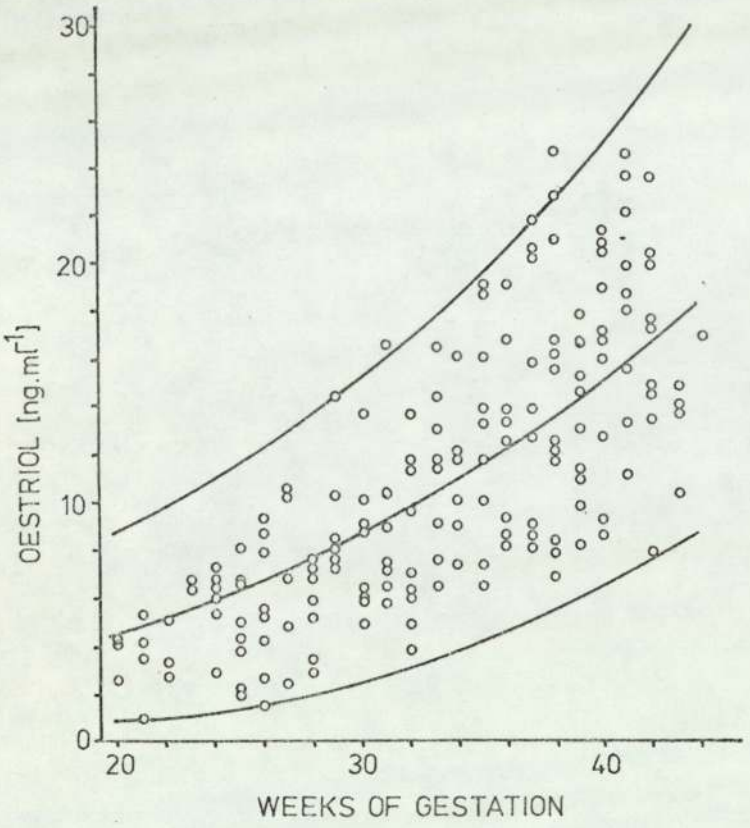



Fig.17:
The levels of serum unconjugated oestriol in 194 pregnent patients. The middle, upper and lower lines represent the mean and 2 SD above and below the mean.
(Data from Cleary et al.[73] ).

considered not to be at risk because the trend showed a steady rise in the levels during succeeding weeks. There is a large element of subjectivity in the importance attached to oestrogen excretion data, and this is due in no small way to the extreme range of normal values. It is my opinion that a large contribution to this range is made by the inadequacies of the assay methods used during the collection of this data, and the reason for this will be discussed later. The causes of reduced oestrogen excretion rates are numerous and it is not appropriate for me to devote much space to these clinical considerations, however it is valuable to mention the more common causes of fetal morbidity (and mortality) which are often associated with endocrine changes which affect the oestrogen concentration of maternal plasma and urine.

Toxaemia of pregnancy, which is a generic term covering a great variety of clinical forms such as hypertension (primary or secondary to the pregnancy) nephropathy or chronic nephritis, is usually associated with reduced oestrogen levels. In mild pre-eclampsia the mean plasma levels of oestradiol are slighty lower than those in the corresponding weeks in uncomplicated pregnancies[229], and the more severe the hypertension or general toxaemia, the lower the oestrogen levels. However, authors are not in agreement on the critical level of urinary

oestriol below which the fetus is in danger. The danger levels quoted range from 12mg. to 3mg. per 24 hours in the last three months of pregnancy[128,221, 404,454] but authors agree that even a slight decrease in oestriol (with time) at this period carries a very serious prognosis at this stage of pregnancy.

Again authors are unanimous on the significance of a sudden fall in the oestrogen value to a very low level for the diagnosis of death of the fetus in utero; a very large number of references for this observation are sited by Jayle[178], and I have found no more recent work to discredit this observation. Advantage can be taken of any sinking of the oestrogen curve to save the child by caesarean section, which should be performed before the fall exceeds 50% of the initial value[26]. In general conditions such as generally retarded fetal growth give rise to low urinary and plasma oestrogen concentrations[229], but, unless the condition is severe, the values often fall within the normal range. The same appears to be true for the plasma oestrogen concentrations in cases of Rhesus-immunisation[229], and urinary oestrogens appear to be even less affected[178]. Some workers have reported marginal differences in amniotic fluid oestriol levels in severe rhesus haemolytic disease[250,361], but in most cases the levels of oestriol were well within the normal range for the appropriate stage of pregnancy, and the test has been found to be of

little clinical use in this condition[266].

Similarly, the reports on plasma oestrogen levels in pregnancies complicated by diabetes mellitus are conflicting. Most of the reports have shown oestrogen fluctuating within the normal range. The oestrone and oestradiol levels have been reported to be low[347] or high[398], and the plasma levels of oestriol have been reported to be low[107,242,251,284,329,347] or normal[107,253,365] or high[223,401]. These contradictions are confirmed by Lindberg et al.[229], where the dominating impression is the lack of a consistant pattern in the plasma oestrogen levels. The whole scale from abnormally high to abnormally low is represented, and this is probably a reflection of the fact that diabetics form a very heterogenous group with large variations in the severity and duration of the disease, especially with regard to differences in renal clearance for oestrogens[92]. The lierature relating to urinary oestrogens is no less confusing, and although it appears that in conditions of severe, uncontrolled diabetes the urinary levels fall on the low side of normal there are no hard and fast rules that can be drawn. With urinary oestrogens there are added complications which make many of the results of investigations subject to doubt. There is very clear evidence that the results of oestrogen estimations obtained by acid hydrolysis of the urinary conjugates are often vitiated by interference from glucose, its derivatives or

ketonic substances[57] . Many of the very low levels reported in the earlier literature[96] must be ascribed to this method of hydrolysis; and even when hydrolysis is effected by enzymic reaction, the sugars present in diabetic urine are still capable of affecting the subsequent assay procedures[48] . Determinations of amniotic fluid oestrogens do not appear to have been made to any large extent, and clinical data is not available.

Hydramnios does not appear to be reflected by decreased urinary oestrogen excretion[162,397,455], but as a general rule, anancephaly is associated with very severe oestrogen insufficiency, probably caused by the atrophy of the fetal adrenals[39] . It used to be the case that the mere observation of an oestrogen insufficiency was enough to cause the instigation of treatment with natural or synthetic oestrogen. Clearly there are still some occasions when this treatment is warranted, but these are probably small in number, and even when this therapy was common practice authorities were divided on its wisdom. In one paper by Dieckmann et al.[101] it is stated that the authors do not think that the regular administration of diethyl stilboestrol to all pregnant women reduces the incidence of early or late abortion or of fetal death; other references support this conclusion[27,327]. However, an equiry by Pigeaud[310] with a group of French obstetricians revealed a considerable degree of

confidence in oestrogen treatment. It is clear that while oestrogen therapy is certainly not indicated in all cases of oestrogen insufficiency there will be instances where it is desirable to exert a luteotrophic action on the corpus luteum of pregnancy or a pharmacological action on the feto-placental circulation. However, the disast rous consequences of the administration of synthetic oestrogens such as diethyl stilboestrol revealed in recent reports[216] ought to make clinicians very reticent about admin--istering this sort of treatment.

## VIII Fetal Monitoring During Pregnancy

As we have already seen, the endocrine balance of the body changes very significantly during pregnancy, and this change can be used to monitor the state and cond--ition of the pregnancy. However, pregnancy also initiates dramatic alterations in many other metabolic processes, and these are frequently deranged in an abnormal gestation. It has long been thought that accurate measurement of phases of these metabolic changes should, logically, be useful in the determin--ation of the status of the fetus, and it has been thought that such data could help to reduce perinatal mortality, and morbidity, and contribute to the solution of the problems of mental retardation, cerebral palsy, and congenital malformation. The literature is replete with the evidence of the clinical usefulness of urinary hormone determinations in the diagnosis, prognosis and management of pregnancy complications[34,144], and there is no need to enlarge on the usefulness, for example, of HCG determination in pregnancy diagnosis, and the value of cytology in the management of threatened abortion. For the assessment of placental function in the later stages of pregnancy the estimation of pregnanediol has appeared to be of distinct promise, but the assay of urinary oestrogens is very much easier and is by far the most popular parameter of fetal wellbeing.

TABLE 2:

| | |
|---|---|
| **ENDOCRINE FACTORS** | |
| CORTISOL | 187, 297 |
| PROGESTERONE | 146, 297, 311 |
| THYROID HORMONES | 328 |
| PORPHYRIN EXCRETION | 196 |
| ALPHA FETO-PROTEIN | 371 |
| HPL | 91, 174, 189, 393 |
| CHORIONIC GONADOTROPHIN | 89, 174, 189 |
| PREGNANEDIOL | 144 |
| OESTROGENS | 73, 91, 174, 188, 297 |
| **PHYSICAL TESTS** | |
| ABDOMINAL MEASUREMENTS | 257 |
| AMNIOTIC FLUID VOLUME | 256 |
| ULTRASOUND | 152, 209, 311, 413, 421 |
| **BLOOD AND SERUM STUDIES** | |
| SERUM ION-BINDING CAPACITY | 428 |
| MATERNAL SERUM PROTEINS | 226, 268 |
| SERUM ALKALINE PHOSPHATATASE | 188, 192, 385 |
| ALKALINE PHOSPHATASE IN GRANULAR LEUKOCYTES | 139, 151 |
| DIAMINE OXIDASE | 109, 391, 392 |
| ROSE-WAALER TITRES | 143 |
| LACTIC DEHYDROGENASE | 45 |
| OTHER ENZYME STUDIES | 262, 334, 356 |
| SERUM COPPER | 160, 298, 451 |
| CYSTINE AMINOPEPTIDASE | 262 |
| B-MSH | 14 |
| FIBRINOGEN | 285 |
| CHOLESTEROL | 343 |
| **PHYSIOLOGIC STUDIES** | |
| RADIOACTIVE SODIUM CLEARANCE | 447 |
| FETAL PHONOCARDIOGRAPHY | 249 |
| FETAL ELECTROCARDIOGRAPHY | 249 |
| FETAL ELECTROENCEPHALOGRAPHY | 342 |
| ATROPINE TRANSFER | 161 |
| METABOLIC CLEARANCE RATES | 34 |
| LEUKOCYTE EXCRETION RATES | 34 |
| MUCOPOLYSACCHARIDE EXCRETION | 405 |
| **AMNIOTIC FLUID STUDIES** | |
| FLUID ANALYSIS IN RH SENSITISATION | 12 |
| OXYGEN AND CO2 TENSIONS | 28, 105, 324, 422 |
| FLUID ELECTROLYTES | 28, 105, 446 |
| FLUID PHOSPHOLIPIDS | 28, 44, 105 |
| FLUID PROTEINS AND AMINO ACIDS | 28, 105 |
| FLUID CREATININE AND UREA | 28, 105, 435 |
| OSMOLARITY | 34, 105 |
| FLUID ENZYMES | 28, 105 |
| AMNIOSCOPY | 354 |
| AMNIOGRAPHY | 260 |
| CYTOLOGY | 28, 105, 185 |
| CERVICAL MUCOUS STUDIES | 366 |

These are methods of fetal assessment that have stood the test of time and continue to be widely used; however, other tests, more recently appeared on the obstetric scene, may well come to be of very much more significance than they are at the present. Table 2 shows some of the parameters that have been investigated for usefulness in the assessment of fetal wellbeing. The list is by no means exhaustive, and the references are representative rather than comprehensive.

Many of the techniques listed are old ones which have been supplanted, and others have been abandoned because they showed little promise. For example, abdominal palpation[257] and measurement of amniotic fluid volume[256] have been supplemented, and to a large extent replaced by diagnostic ultrasound methods[152,209,311,413,421]. It appears that vaginal hormonocytology[28,105,185], pregnanediol excretion[144] and a study of enzymes[28,105] appear to be valuable in the assessment of the status of early pregnancy. The use of amniocentesis with subsequent examination of the amniotic fluid is at present one of the best ways to assess fetal wellbeing in those pregnancies complicated by Rh isoimmunisation, however, recent work[140] has raised the question of whether this is a fetal investigation or fatal investigation, following the death of several fetuses in this, still hazardous, procedure. In other complicated pregnancies, the measurement of urinary oestriol excretion rates can

determine the condition of the fetus[42,73,91,174,188,297] and this, together with HPL and HCG assays remains the most popular technique in the country (and also in the USA).

In general there are three comments to be made about the current scene of fetal assessment. Firstly, the continuing predominance of oestrogen assays; secondly the high level of interest that has been maintained in ultrasonic methods; and thirdly, the changing emphasis that can be seen in the literature of the last couple of years in regard to several tests, particular HCS/HPL tests, and assays involving amniotic fluid. The large number of papers published on oestrogen assays[34] shows the reliance which is placed on this test of fetal wellbeing and develop--ment. Almost all of the newly described methods of fetal assessment have been compared with urinary oestrogen assays, and the oestriol assay has become the yardstick against which all other tests are measured.

## IX Determination of Oestrogens

### A. Isolation from Body Fluids and Tissues

As has already been mentioned, the assay of oestrogens in order to determine their concentration in the various fluids and tissues of the pregnant woman is of dominant importance in the assessment of the wellbeing of both mother and child. It is clear that there are countless other situations where it is neccessary to be able to determine, with accuracy, the levels of oestrogens in a wide variety of chemical and biological mixtures. The very great diversity of extraction and assay techniques which have been developed for this purpose testifies to the importance which has been placed on this assay.

The problems of isolation may be various, and of differing degrees of complexity. For instance, it may be neccessary to isolate a particular phenol steroid from a pharmaceutical preparation (anti-inflammatory cream or contraceptive pill for instance) where the extracted compound has to be identified accurately and be distinguished from (closely related) accompanying molecules; or the oestrogens from a mares urine may need to be extracted in order to serve as a starting material for preparative chemistry; in this case the isolation may need only one step in order to yield a correct fraction of steroids in the required purity. It is to accomodate this range of requirements that the

wide variety of separation techniques has been developed.

The most commonly used techniques depend on solvent solvent partition phenomena, or on column chromatographic stages. Below, I have described some of the more popular techniques and have given representative references for their use.

The most extensive use of column chromatography seems to have been with the various forms of gel filtration materials. The early work by Beling[24] showed that Sephadex G25 could be used to separate conjugated oestrogens from urine, and resolve some of the components of the mixture. The separation is not a true gel filtation (separation on the basis of molecular size) but appears to be a function of the ionic strengh of the elution fluid. Urine is applied to the column, and the components eluted with distilled water, there is thus a continually decreasing ionic strength to the eluting fluid, which causes a fractionation of the steroid conjugates. Adlercreutz[3] studied the elution fractions of this method and found that glucose and D-glucosaccharic acid, as well as a large number of other impurities were eliminated from the oestrogen fractions by the gel filtration, and that the method was a 'great advance in oestrogen methodology'. It was also noted, in the same report, that differing quantities of B-glucuronidase were needed to hydrolyse the conjugates eluted in the different fractions. Because

of their insolubility in water and small size, the free oestrogens are not suited to chromatography on the (aqueous) molecular sieves, but the Sephadex LH types of gel are hydroxypropylated cross-linked dextrans which swell in organic solvents, and these have been used with some success to separate free oestrogens other steroids in mixtures[67,267] . However, the aqueous gel types (G25 and G50) have been used to seperate free oestrogen from protein bound oestrogen in plasma samples[121] . Using a technique called Steady-state gel filtration (SSGF), a technique which maintains physiological conditions of pH, temperature and plasma constituents, thus avoiding alterations in the original equilibria, it has been possible to measure the percentage of unbound oestradiol in a plasma sample[121]. When plasma is applied to a Sephadex column, molecular sieving of the unbound steroid causes the leading edge of the sample to become progressively stripped of steroid as it advances down the column. Fresh plasma sample then encounters this dissociated steroid migrating down at a slower rate, so that net dissociation diminishes and finally a new equilibrium is set up. If the applied sample is sufficiently large the dissociated steroid concentration in the gel eventually becomes equal to the unbound steroid concentration in the plasma sample, preventing any further dissociation, ie. a steady state is reached. Elution at this point with buffer gives a plateau

containing the plasma proteins and their steroids at their original concentration, followed by a second plateau corresponding to the unbound steroid fraction retarded by the gel.

Sephadex G10 has been used to isolate oestrogens from plasma[72], the extracted steroid being equilibrated, subsequently, on the same column with oestrogen antibody protein then eluted to fractionate protein bound from free oestrogen as a preliminary to radioimmunoassay (RIA). Sephadex LH20 has been used in a similar way[399,424], and has also been used to separate oestriol from oestradiol and oestrone in ether extracts of these steroids[453], again as a preliminary to RIA.

Celite columns have been used to isolate oestrogens from contaminating steroids, the mixtures being dissolved in iso-octane and eluted from the column with mixtures of iso-octane and ethyl acetate[76,337,360] Celite has uses in some plasma assays too, having been used in an assay of ethinyl oestradiol in (for example) oral contraceptive formulations[448]. Non-oestrogenic steroids and tablet excipients in samples interfere with the colorimetric determination of ethinyl oestradiol with the mwthanol-sulphuric acid reagent used in this test, and so a separation stage was required before colour formation. A sodium hydroxide trap layer in the chromatographic column was found to retain ethinyl oestradiol as its phenolate salt while the extractable, interfering

non-acidic tablet components were washed off with chloroform-isooctane solvent mixture. The oestrogen was subsequently eluted with chloroform.

Ion exchange chromatography does not appear to have been used to any great extent in the separation of oestrogens. Adessi[1] used an anion exchange resin (AG 1x2) to purify hydrolysed urinary oestrogens prior to GLC, and claimed a very high degree of purification and concentration. The approach is new, and may well come to be of considerable importance as further work is devoted to this method of fractionation. However, disappointing results in the chromatographic separation of steroid conjugates have been reported[148] for anion exchange resins with hydrophobic polystyrene-divinyl-benzene matrixes, probably because of interactions with the steroid skeleton. Recent reports of the application of High Pressure Liquid Chromatography (HPLC), with a range of anion exchange resins, to the separation of oestrogens[418] suggests that this approach will come to be of greater importance in the future, as may, for example, the separation of equine oestrogens by High speed Liquid Chromatography[64].

Paper chromatography has been used to separate oestrogens, but with urinary extracts the conjugated steroids usually need to be hydrolysed before separation is undertaken. It is generally more difficult to separate a given pair of steroidal glucosiduronic acids by partition chromatography than it

is to separate the respective pair of parent steroids. A chromatography system which gives a satisfactory partition coefficient with a free steroid will need to have the polarity of the mobile phase increased before it will give a useful coefficient with the corresponding steroidal glucosiduronic acid, this increase of the polarity of the mobile phase decreases the difference in the polarities of the mobile and stationary phases, a change which often reduces the selectivity of a chromatography system. However this problem has been partially overcome by the use of liquid ion exchangers such as tetraheptylammonium chloride (THAC)[255].

With free oestrogens, paper chromatography at 32° using a solvent system of light petrol and methanol has given satisfactory results[270], the spots being detected either by their UV absorption or by colour reaction to Folin & Ciocalteu's reagent[238]. A number of the early solvent systems and operating conditions are given by Bush[63] and these are still used quite extensively with little modification. When very labile oestrogens, such as the 2,3-dihydroxy oestrogens, are chromatographed on paper, there is the risk of a very high rate of decomposition unless adequate precautions are taken. With these compounds it has been found advisable to include reducing conditions in the chromatographic procedure[132], for example by introducing ascorbic acid into the stationary phase. Partition chromatography is normally

used in conjunction with other techniques during extensive purification methods[23], and is not involved to any great extent in the rapid determinations of oestrogens extracted from urine and/or plasma.

Thin-layer chromatography (TLC) on silica gel has been used in the selective extraction of oestrogen glucosiduronic acids from aqueous solution with a chloroform-isopropanol solvent mixture[124], the subsequent purification being by two-dimensional migration on thin plates using chloroform, isopropanol and formic acid as solvents. A similar technique has also been used for the isolation of hydrolysed oestrogens from urine[115], and from fetal tissue[402] in which the support was Kieselgel G and the solvent system was benzene-ethanol (9:1). Oestrogens can be detected with a spray of 20% toluene-p-sulphonic acid in ethanol, which gives an orange-yellow stain with oestradiol and oestrone[426].

Gas-liquid chromatography (GLC) has been used very extensively in the purification and assay of oestrogens. Direct injection of blood or urine into a gas chromatograph, such as has been described for the determination of alcohol concentrations in blood[172], has not yet been achieved successfully for the determination of steroids; therefore extraction of steroids from urine or blood samples is required. The use of very crude extracts can lead to distortion and overlap of peaks[417] therefore sample purification prior to GLC is often required; however, the time

and trouble spent on sample preparation for GLC will be worthwhile if the sensitivity and/or specificity of detection cannot be matched by other techniques. Reliable methods using GLC with electron capture detection[56,325] and flame ionisation detection[2,388] have now been published for the measurement, in biological samples, of nanogram amounts of oestrone, oestradiol and oestriol. In these instances, both separation, identification and estimation are carried out at a single step. However, the specific isolation of steroids, using GLC, followed by further character--isation of the steroids using techniques such as mass specrometry[4,6] has lead to the identification of a large number of oestrogen derivatives in urine and blood. Typical column materials are Gas Chrom Q with 3% OV-1[388] or OV225[2] . In general it is true to say that GLC combines a highly efficient separation with a very sensitive quantitative detection. The efficiency of GLC columns is demonstrated by the separation of ring B hydroxylated phenolic steroids[56]. By the use of an electron capture detector (ECD) a very high sensitivity is achieved; however, only halogenated derivatives of steroids are suitable for gas chromatography with the ECD.

More recently, the combination of GLC with mass spectrometry has become of great value in the deter--mination and analysis of steroids such as oestrogens. Two possibilities for the use of this combination exist. Firstly, by scanning the mass spectra of the

eluates obtained after gas chromatography it is not only possible to identify known or unknown steroids, but the specificity of the gas chromatographic method may also be checked. Secondly, the mass spectrometer may also be used as a highly specific detector for GLC. This is done by setting the mass spectrometer to characteristic m/e values, using a multiple ion detector. The continuous recording of the m/e values yields gas chromatograms which specifically show only those substances whose mass spectra have the appropriate m/e values[56].

Typical derivatisation reactions, to increase the volatility of the oestrogens, include acetylation[388], reaction with dansyl chloride[290], reaction to yield trimethylsilyl ethers[120] or with bis-(trimethylsilyl)-acetamide-trimethyl chlorosilane[1]. A number of other derivatives are listed by Rajkowski[325].

The clinical data collected using GLC oestrogen determinations is still not comprehensive. Lockwood et al.[234] have published data concerning the urinary oestrogens in pregnancy, but GLC seems to have been used as a more accurate and sensitive research tool than as a routine screening method. This may reflect a reticence on the part of the hospital laboratories, or may result from difficulties these laboratories have experienced in establishing reliable techniques based on a GLC method. There appear to be less than 1% of hospital labs. using GLC as a method for the determination of oestrogens in pregnancy urine[292].

Very recently there have been reports of the application of pyrolysis GLC to sterols and steroids[130], although this technique has not yet been applied in any clinical situation to oestrogens. In this approach, thermal decomposition products are liberated into the GLC column as well as the volatilised steroid, and the resulting spectra are often more complex than those from a standard derivatised oestrogen solution, but there is the very great advantage that derivatisation reactions, with their attendant loss of material, are avoided. There may not even be a need to isolate the oestrogens from urine/blood prior to their analysis if pyrolysis GLC/Mass Spec. is adopted[148].

Isotopically labelled steroids make an important contribution to the assay and determination methods available for oestrogens[20,75]. Gas-liquid radiochromatography has been used with oestrogens[389,390] where the radioactive eluant from a GLC column is detected, trapped and recognised by virtue of its radioactive emission; but by far the most important use for labelled steroids now is in radioimmunoassay procedures which have pushed forward the limits of sensitivity to levels which cannot be reached by chemical or physical means. This approach will be discussed in the next section.

Historically, the most important technique used for the separation of oestrogens prior to assay in a clinical environment is solvent-solvent

partition chromatography, or straight solvent extractions[4,78,81,167,254,270,293,345,387,416,420]; and this method is still used, even in the most recently published assay procedures[47]. Despite this pedigree, however, there remain very few different approaches, and the basic methodology has remained unchanged for nearly twenty years. For urinary oestrogens, the urine is first subjected to acid or enzymic hydrolysis, to leave free oestrogens, then the urine is extracted with ether, this ether extract being extracted with a bicarbonate solution prior to assay of the dissolved oestrogens[47,345,416]. Some authors have preferred to extract the oestrogens with chloroform[416] and others have introduced extra solvent extraction stages in order to remove non-phenolic steroids; but the majority of methods which use this general technique remain very simple and uncluttered. Soxhlet extraction has been used[387], and this undoubtably gives a more exhaustive extraction than simple mixing and agit--ation, but, where time is short, workers have preferred to sacrifice a (small) degree of sensitivity in favour of more rapid results. The mechanics of some solvent extraction procedures will be discussed in Section X.

Precipitation reactions using either charcoal, charcoal/dextran or ammonium sulphate[4,68,77,301,312,432] have been used to isolate oestrogens and oestrogen conjugates from plasma and urine, and simple adsorption chromatography has also been used[17,58,88,206]

typically to isolate protein bound oestrogens from plasma.

It has been found that a very quick, distinct and reproducible separation of steroid glucuronides from steroid sulphates, as well as steroid monosulphates from disulphates, and monoglucuronides from diglucuronides can be achieved with the aid of high voltage electrophoresis on paper[207] but this technique has not found great favour in the clinical situation. In this sector, much more effort has been expended in establishing automated methods of oestrogen assay[168], and these are now becoming standard in many hospital laboratories. Most of these methods depend on a fluorimetric parameter for estimating (urinary) oestrogens. A comparatively new development, which is unlikely to be applied routinely for some time, is the application of 'artificial intelligence' to analyse mixtures of (for example) oestrogens without prior separation[384] this is done by the computerised interpretation of a variety of mass spectral data aquired on underivatised, unseparated mixtures. This data includes high resolution mass spectra, low ionising voltage spectra and metastable ion spectra. Clearly this approach is beyond the scope of most research laboratories, let alone hospital laboratories, but it may well point the way future trends will lead.

B. Assay Methods for Oestrogens

The tremendous variety of methods available for the assay of oestrogens from biological fluids is, in part, testimony to the clinical significance of oestrogen assays in the management of pregnancy. Some of the methods have not really gained a great deal of popularity and are of somewhat academic interest. Clearly, identification and quantification by mass spectrometry[4,6] finds its major application in the research laboratory rather than in the clinical sphere; and the results gained from this technique can be extremely illuminating if metabolic studies are involved, but for routine use it has little to offer. Field desorption mass spectrometry has been used with oestrogen conjugates as a means of obtaining accurate molecular weights[5] . The molecular ion peak of the underivatised conjugate can be obtained quite easily using the gentle conditions of FDMS.

Fèher and Csillag have used TLC methods to isolate oestrogens from pregnancy urine[115], subsequently scraping away the spots from the plate and assaying their contents, but again, this is hardly suited to routine applications.

Biochemical oestrogen assays are of great value in determining whether a particular compound is an oestrogen or not. Several have been developed[179], and some have claimed to be quantitative, although

no serious attempt could be made to assay known oestrogens using this sort of approach.

The oldest approach to the problem of oestrogen assay which still has application now is the colorimetric determination method. The first colorimetric assay method for oestrogens in pregnancy urine was that of Cohen and Marrian[79] who in 1934 applied the Kober[204] colour test to an ether-soluble, phenolic fraction of acid hydrolysed urine. Some twenty years later this method was modified by Brown[58] to increase its sensitivity thus permitting the application of the test to non-pregnant urine. In this procedure, the hydrolysed oestrogens were extracted from the urine by ether, and the ether phase was washed with alkali to remove coloured contaminants from the organic extract. Much of the material in this extract is not phenolic, so a reaction with dimethyl sulphate was included to form methyl ethers of the phenols. These ethers are very insoluble in alkali, so the extract was taken to high pH, and reacted with hydrogen peroxide to oxidise non-phenols to alkali-soluble products. The mixture was then extracted with benzene (oestriol ether) and light petrol (oestrone and oestradiol ethers). Colour development was by reaction with sulphuric acid/quinol reagent, followed by heating with sulphuric acid for about 10 minutes. The colours produced were stable for at least 12 hours and absorbed light maximally at about 515 nm.

In 1958 Ittrich[171] showed that the intensity of the colour, and the selectivity of the test, were increased when the colour was extracted with a solution of p-nitrophenol in a chlorinated or brominated organic solvent. However, at this stage the assay could take anything from 1 to 3 days for the accurate assay of about six samples of urine. With the advent of feto-placental monitoring, and the increased interest in oestrogen assays it became important to provide very rapid assays, and this remains one of the prime objectives of workers in this field.

Cohen, in 1966, reported a revision of his earlier method, in which he claimed to be able to complete six assays in 4-5 hours[77] . In this method the Ittrich colour reaction[171] was applied to a simply purified, unhydrolysed urinary residue. Purification was effected by quantitative precipitation of the urinary oestrogens with 70% (w/v) ammonium sulphate. In his paper,[77] Cohen reports values for oestrogens in pregnancy urine which are 44% higher than the values determined (on the same samples) by the Brown method, these differences being attributed to the degradation of oestrogens by the caustic conditions employed by this latter technique.

Ittrich had shown in his paper[171] that the extracted colour was also fluorescent, and that this fluorescence could be used as the basis for the determination of the oestrogens. Because fluorescent

techniques are inherently more sensitive than those based on absorption or colorimetric procedures, this observation provided a way to increase the sensitivity of the assay without altering any of its experimental conditions. The sensitivity is limited, usually, by the background emission; that is, the fluorescence of residual impurities present in the extract. Corrections to allow for these impurities were intro--duced in 1962[346] and 1964[239] based on the Allen corr--ection[60] applied to the emitted light. The mechanism of the Kober reaction has been investigated recently[194] and it appears that the Kober chromophores are constituted by resonance-stabilised carbonium ions of the steroid, and that a dehydration in ring D by the sulphuric acid is essential in initiating the reaction. There are an immense number of reaction products from even quite pure oestrogen extracts[194] and when the reaction is applied to plasma or urine samples, or to pharmaceutical preparations of oestrogens, then it is found that non-oestrogenic steroids or tablet excipients in the samples interfere with the colorimetric or fluorescent determination of the oestrogens[448]. Nevertheless, very many color--imetric methods continue to be used, which have the Kober reaction as their basis[65,82,167,345,387], and two of the most popular methods in use today, the color--imetric assay developed by Oakey[293] and a closely related fluorescence assay by Brombacher[57] are used

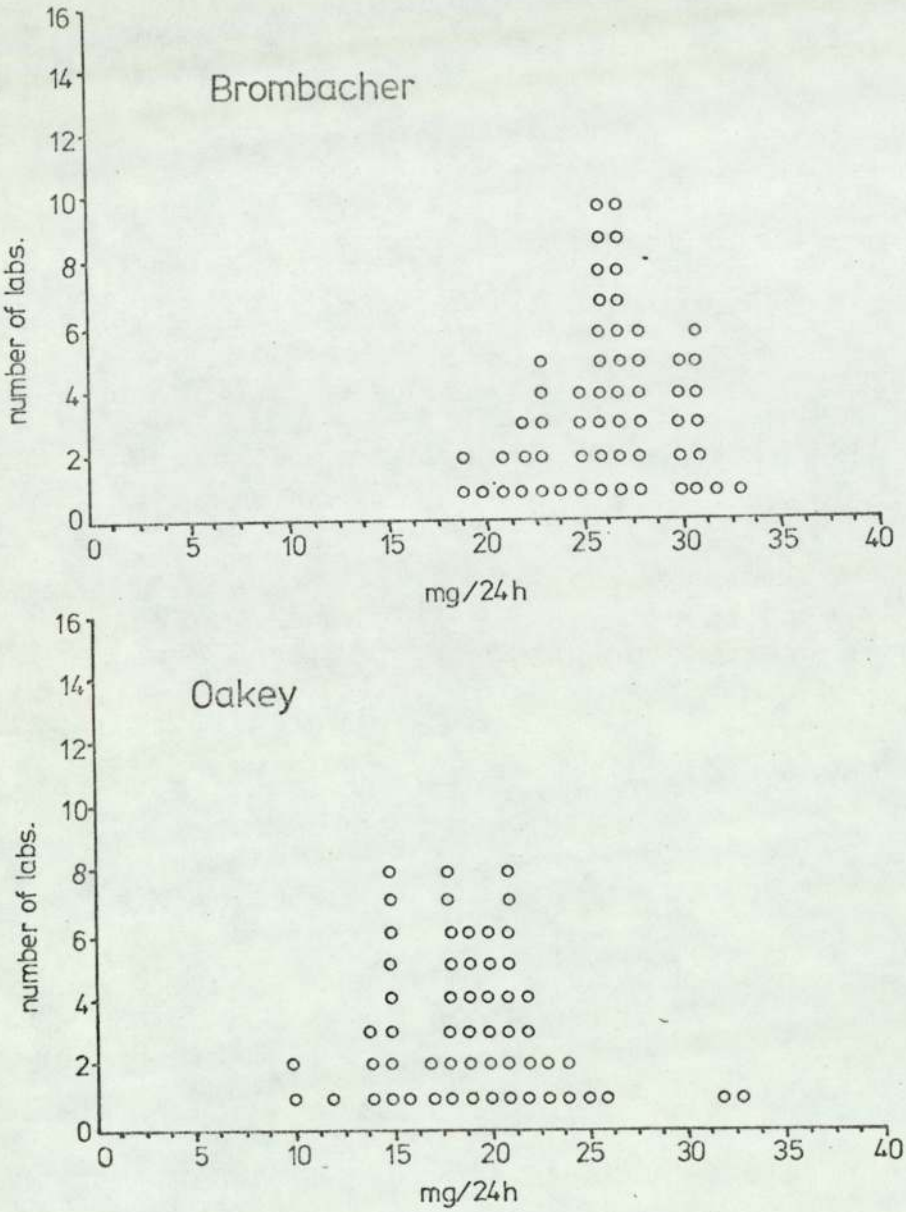



Fig.18:

Results, from 102 hospital laboratories, for the determination of oestrogens in a sample of pregnancy urine by either Oakey's method or Brombacher's method. (Data from National Control Sample 20 viii.74).

in about 70% of hospital laboratories currently providing a service of oestrogen assays[292]. Fig.18 shows how these two assay methods give very different impressions of the amount of oestrogen present in a sample; and even more disturbing than the inter-assay variation (the Oakey assay gave a mean value of 19.14 mg per 24 hour with a standard deviation of 4.33, and the Brombacher method gave a value of 27.38 mg per 24 hours with 4.19 SD, which a variation which might be expected between a colorimetric and a fluorescence assay) is the very wide range of values obtained by the different laboratories using the same methods[292].

A number of other assays have been described using the fluorescence of the Kober chromophore as the parameter of oestrogen concentration. Some of these, like the Brombacher technique, have used the Kober-Ittrich method in a virtually unchanged form[57,270] whilst others have used slight variations such as the addition of tetrachloroethane[254] or tetrabromo--ethane[312] to the assay mixture which enhances and stabilises the fluorochromes. A recently published assay of oestrogens which uses the substrate native fluorescence, and which thus circumvents the problems of the Kober reaction[47] is discussed in Section X

The use of GLC for the assay of oestrogens has already been mentioned in Section IX. Potentially, this approach has much to commend it because of the

high resolution which can be obtained of (quite complex) mixtures of steroids, and also because of its great sensitivity. However, the main drawback is the need to derivatise the oestrogens prior to chromatography. The derivatisation step may only be a one-stage reaction, but it will always procede with less than 100% yield, and so will always intro--duce a decrease in the sensitivity of the overall assay In addition, it is usually required that the steroid fraction be free of contaminating substances prior to the derivatisation step, which necessitates time-consuming extraction and clean-up procedures. This problem can be circumvented if pyrolysis-GLC is used[130], but this approach has not yet found real favour in the hospital sphere. There are a few assays which use GLC determinations of oestrogens in plasma samples[2,23,120] and in urine[388] but these are not widely used, partially because of the reasons noted above, and partly because of the need to have a dedicated range of equipment (which cannot easily be used for other purposes) and the services of skilful and competent technicians without whom any GLC approach is doomed to failure. The major attraction of the technique is its ability to detect and assay the low levels of oestrogens which are associated with non-pregnancy plasma or urine, but even here, GLC methods are overshadowed by the vastly more sensitive RIA and competitive-protein-binding (CPB) methods.

With these methods, oestrogens are extracted (typically from blood) with an appropriate organic solvent such as diethyl ether. This organic extract is then purified, usually on Sephadex LH 20[399]. However, LH 20 chromatography is time-consuming and requires considerable skill. For clinical measurements the use of antibodies with a high specific activity can reduce the need for a preliminary clean-up of the oestrogen extract[228]. The oestrogen fraction is then mixed with a known amount of radiactively labelled oestrogen, and the mixture is added to a solution of a binding protein or of an antibody to the oestrogen[68,190] The mixture is stirred and allowed to equilibrate, then the oestrogen which is bound to the protein is separated from that which is still free, usually by absorbing the protein onto dextran-coated charcoal and collecting the precipitate[76,88,206,228,301,337,424,453], but sometimes by precipitating the protein with polyethylene glycol[360] or retaining it on a column of Sephadex G10[72]. Dextran-coated charcoal has always been used for the separation of free and bound oest--rogens without any obvious indication that the steroids are stripped from the hapten-antibody complex, but Lindberg and his colleagues[228] have shown that there is quite a pronounced stripping effect which, although it may not affect the accuracy of the method, will certainly decrease its sensitivity. These authors preferred to use saturated ammonium sulphate to precipitate the protein complexes, and claim that

any stripping which occurs with ammonium sulphate is of only minor importance.

If a CPG method is used, the protein can be extracted from the blood of any appropriate animal SHBG (see Section VI ) is used very often for this purpose and it can be prepared from human late-preg--nancy plasma quite easily[399] . Most RIA methods, on the other hand, require the production of an approp--iately specific antibody protein. Typically, the protein is obtained from rabbits by the injection of conjugated oestrogens. In this case the oestrogen in question is converted into a form which can subsequ--ently be attached to an (inert) protein such as bovine serum albumin (BSA). For this purpose a very wide range of reactions has been developed, and these have been reviewed by Lindberg[228] . Succinate and hemisuccinate esters have been the most popular conjugates to date, but oximes such as oestriol-ó--(O-carboxymethyl) oxime or oestriol-4-azobenzoic acid[190] have also been used to link the oestrogen with the protein. Once formed, the protein/oestrogen complex is injected into a willing rabbit, which is then allowed to develop antibodies to the antigenic stimulus of the oestrogen-BSA. The antiserum thus developed is responsive to oestrogens, whether free or protein-bound, and can be used in the RIA. Antibody raised after the injection of oestrogens conjugated to BSA through the 17 position of the

steroid ring system cannot easily differentiate between oestradiol-17a and oestradiol-17B, oestradiol 17B-D-glucosiduronate or oestrone, each of which have their determinant configurations in the vicinity of the site for conjugation[228]. On the other hand, anti--body raised after conjugation in the sixth position will easily recognise most of the oestrogens with determinant structures at C-16 and C-17, but less well oestradiol-17B-3-benzoate with a determinant position at the 3rd position in the vicinity of the C-6.

Most of the neccessary components for a RIA assay can now be bought commercially, and several 'kits' are on the market which reduce the amount of basic preparatory work to an absolute minimum. However, all RIA kits are expensive, not only because of the cost of the reagents used, but also because of the expense of mantaining suitable laboratories as radiation zones, and of the cost of training and protecting technicians who have to carry out the assay. Without a doubt, when very low levels of oestrogen need to be assayed, and this is often the case with plasma determinations, the RIA is unparalleled; there is no other technique so sensitive and accurate. With urinary assays the situation is rather different. Here the levels of oestrogens are very much higher, and in the case of late/middle pregnancy, the levels are astonishingly

high. It is quite irresponsible to use RIA for the determination of oestrogens in pregnancy urine, not only because of the needless expense to the Health Service as a whole, but also (and much more importantly) because of the needless contamination of the environment with radioactive wastes of various sorts. Most hospital laboratories will come to have adequate counting facilities as the use of RIA becomes more and more widespread, but it is my opinion that there is no justification for the use of this technique in the determination of urinary oestrogens in the management of pregnancy.

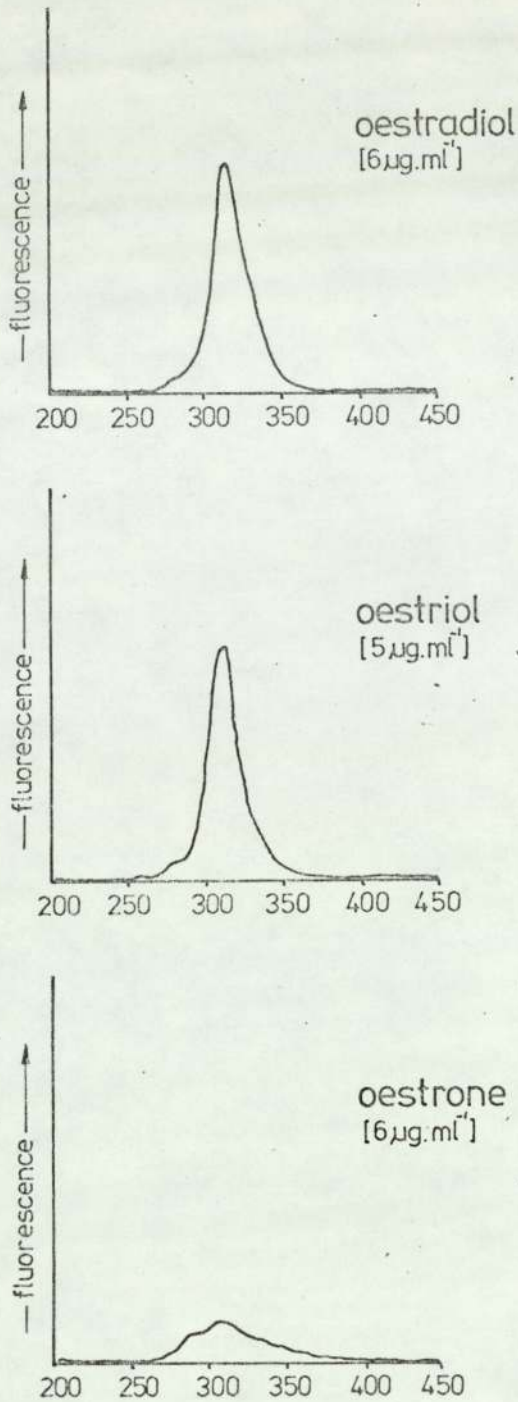


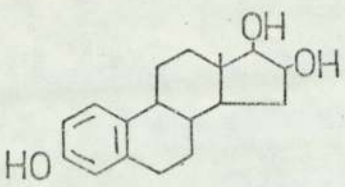

Fig.19:

Fluorescence emission spectra of oestrogens.
[Ordinate: intensity in arb., but consistent units; abcissa: wavelength in nm]

## X A New Assay for Oestrogens

Without exception, all the methods currently available for the determination of urinary oestrogens involve a high degree of technical skill, occupy many hours of laboratory time, and - in many cases - yield results which are so variable and inconsistent as to call into question their value as diagnostic aids. As has already been observed, the involvement, in any assay, of strongly acidic conditions (such as those found in the acid hydrolysis of urine, or the development of Kober chromogens) will render the results of that assay much less reliable than they otherwise could be, and the development of chromophores by chemical reaction is bound to have an attendent loss of overall efficiency of the assay, which can be ill-afforded.

Since the oestrogens commonly found in pregnancy urine are all phenolic, it seemed likely that, as phenols, they might display native fluorescence if excited by light of an appropriate wavelength. Native fluorescence had already been demonstrated for oestrone and oestradiol[433] and the emission spectra in Fig.19 confirm this observation. The spectra were obtained using an Aminco-Bowman spectrofluorimeter equipped with a 250W xenon arc lamp, a source which approximates daylight very closely. The photomultiplier was an EMI type 9781B which shows a modified S-5 response (i.e. is sensitive to radiations between 185 and 650 nm). The response profile of this photomultiplier



| excite λ | emit λ | quantum yield |
|---|---|---|
| 282 | 302 | 0.22 |
| 280 | 307 | 0.008 |
| 284 | 307 | 0.20 |
| 282 | 302 | 0.17 |
| 282 | 302 | 0.008 |
| 284 | 307 | 0.20 |
| 282 | 302 | 0.10 |

Fig. 21:
Fluorescence characteristics of oestrogens.

tube is shown in Fig.20. The fluorimeter was equipped with diffraction gratings which were blazed at 300 nm, and the fluorescence spectra obtained from the instru--ment were corrected for the variation of instrumental response with wavelength by using a computer program: 'FLUCORR'[233]. With this program, the spectrum to be corrected was entered in free format, and outputted as corrected energies at incremental wavelength values.

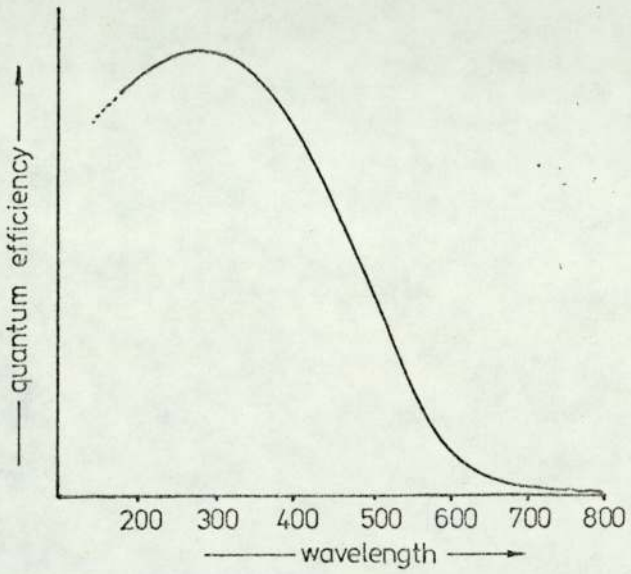



Fig.20:
Response profile of EMI photomultiplier, type 9781B, used in this work.

Figure 21, opposite, shows the excitation and emission maxima of several oestrogens studied in ethanolic solution. The figure also shows the approximate quantum yields of the molecules. The quantum yield of fluorescence has been defined as the fraction of molecules which emit a photon after direct excitation of the source[95]. With a 250W xenon arc lamp, with

relatively low intensity after monochromation, there is quite a low probability of multiphotonic absorption, and so the assumption can be made that, under these conditions, the excitation of a molecule is a single photon process, and the quantum yield becomes the ratio of the emitted to absorbed photons. Thus, to determine the quantum yield, both fluorescence emission intensity and absorption measurements must be made. Absorption measurements were made using a Beckman Acta V absorption spectrophotometer; and it was considered that this instrument gave absorbances to 0.005 or better. Thus a 5% accuracy was achieved by using solutions of absorbance greater than or equal to 0.10; and, to minimise concentration effects, the absorbances were kept as low as possible above this value.

It has been shown that, comparing two compounds:

$$\frac{F_2}{F_1} = \frac{Q_2}{Q_1} X \frac{A_2}{A_1}$$

where A is the absorbance, Q is the quantum yield, and F is the fluorescence of the two compounds 1 & 2 res--pectively[307]. Thus, the quantum yields of the oest--rogens were obtained by comparison of their absorp--tion and emission data with similar data collected for a substance whose quantum yield is known accurately. Compounds used as standards in this way were tryptophan, 5-indanol and p-terphenyl.

Oestriol and oestradiol have very similar fluorescence and absorbance characteristics (and hence

Ordinate: Log fluorescence emission intensity

Abcissa: Oestradiol concentration (log $ng.ml^{-1}$)

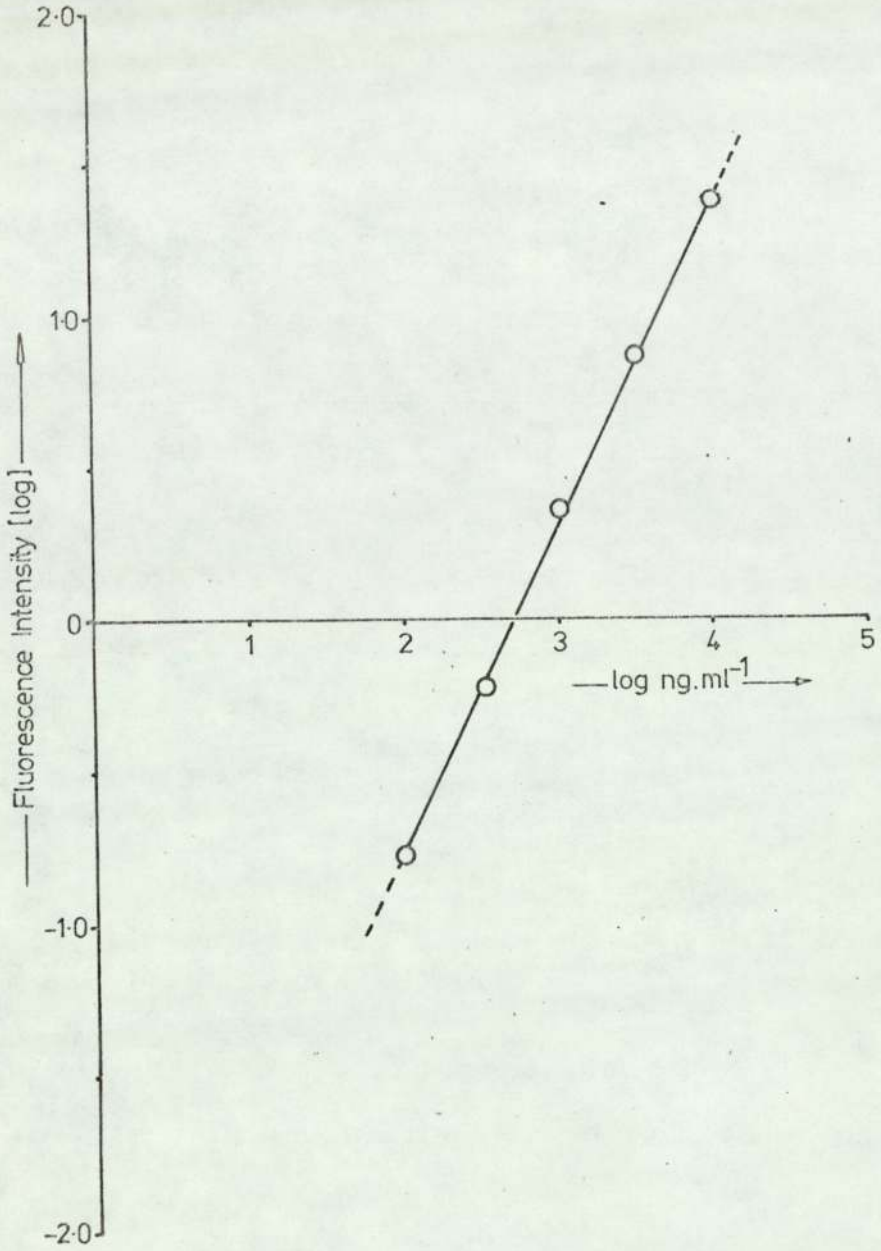



Fig.22:

Relationship between solute concentration and fluorescence emission intensity for a solution of oestradiol-17B in ethanol (log:log scales).

very similar quantum yields). Oestrone shows a remarkably diminished quantum yield of conversion, which is attributable to the ketonic function at position 17. The predominant chromophore of the oestrogens is the phenolic A-ring. The $\pi$ to $\pi^*$ transitions of this system give rise to the intense absorption seen at the short wavelength of 280-290 nm, with the excited electron being delocalised over the entire chromophore. It is the decay from this excited state which constitutes the emission spectrum of the oestrogens. The oestrone molecule, however, also possesses a carbonyl function and this is capable of a $n$ to $\pi^*$ transition, which is of longer wavelength and lower intensity than the phenol. It is this chromophore which is undoubtably responsible for decreasing the apparent emission from the phenolic function in oestrone. The quenching appears to be a long-range interaction across the molecule[33], since there is no trivial quenching observed when a solution of oestradiol is contaminated with oestrone. Because the amount of oestrone in pregnancy urine is proportionally so small as to be neglected, and since the presence of oestrone did not appear to modify the emission qualities of oestrogenic co-solutes, this low quantum yield of oestrone did not present any threat to the establishment of an assay for oestrogens by direct fluorimetry. Fig.22 shows that range of concentrations of oestradiol over which fluorescence

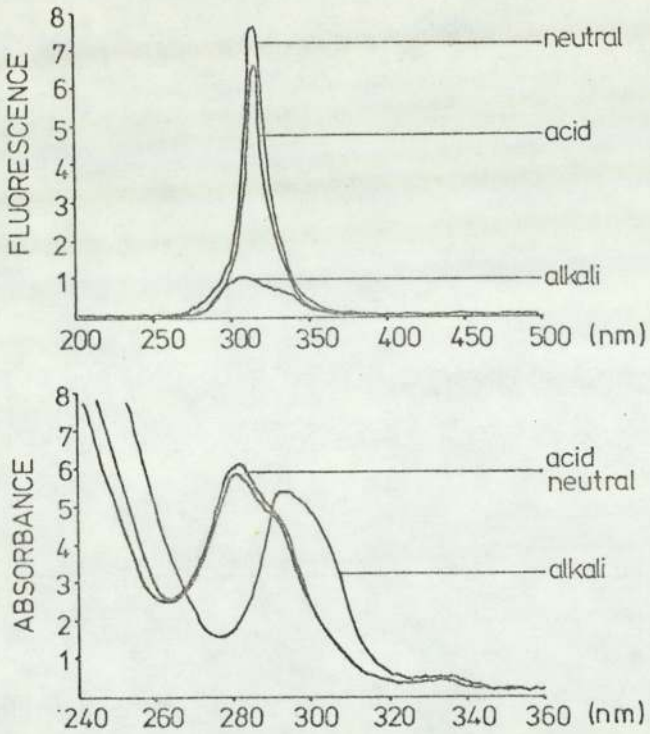



Fig.23: Aqueous oestradiol-17-glucuronide
Effect of pH.

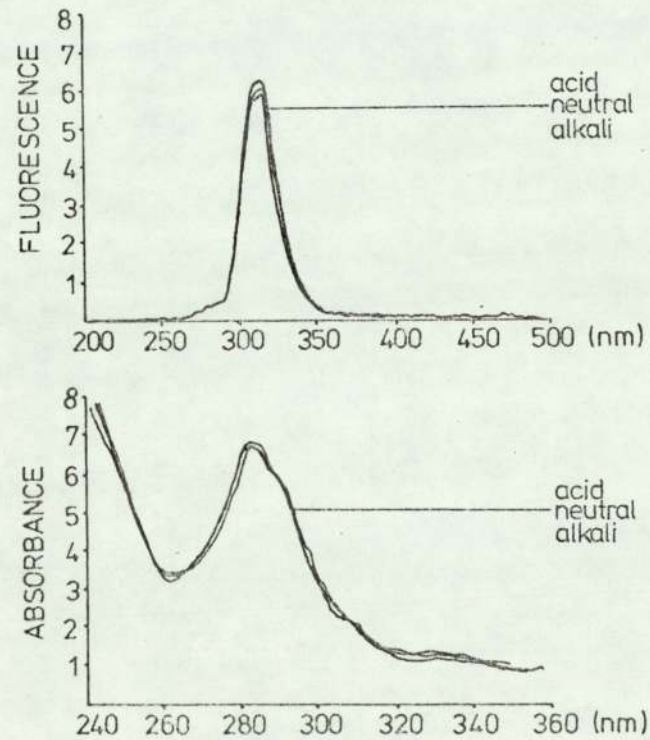
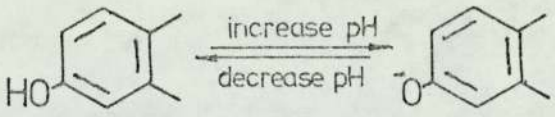



Fig.24: Aqueous oestradiol-3-glucuronide
Effect of pH.

emission intensity is directly proportional to solute concentration. It is clear that, if the fluorescence emission intensity lies within the range of linearity, then intensity of fluorescence may be taken as a direct parameter of oestradiol concentration. In other words, if the emission of a solution of oestradiol is measured, and found to be within a certain range, then the concentration of oestradiol in that solution can be calculated. This observation forms the basis of the assay for oestrogens in pregnancy urine.

Pregnancy urine is, to an approximation, an aqueous solution of oestrogens. Since about 95% of urinary oestrogen is conjugated with glucuronic acid[135] it is clear that conjugated oestrogen determinations are an extremely close approximation to total oestrogen determinations. These conjugates themselves show fluorescence characteristics which are remarkably similar to those of the free oestrogens (Figs.23 & 24). It is interesting to note the effect of alkaline conditions on the emission from the 17-glucuronide. This effect of pH on the spectral responses of the two oestradiol conjugates casts light on the behaviour of the free oestrogens in aqueous solution. The nor--mally phenolic oestrogens exhibit a bathochromic shift when the environment is made more alkaline because of the dissociation of the alcohol function at the 3-position. This dissociation results in the formation of the phenoxide ionic form of the steroid,

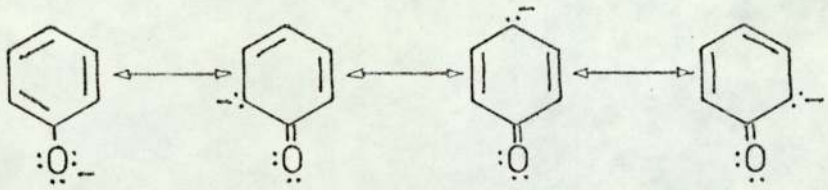
as shown in Fig.25 below:




phenol phenoxide

Clearly, this behaviour is prohibited when the steroid is conjugated through the 3-position. The relatively acidic properties of the phenolsteroids is attributable to the resonance of the phenoxide ion, shown below in Fig.26:

Fig.26:



The negative charge is not resident on the oxygen atom, but is delocalised over the entire A ring. This results in a low electron density on oxygen, a low basicity of the negative ion, and a high phenoxide concentration at equilibrium.

The resonance is analogous to the principal resonance cont--ributers of the carbonyl group, shown in Fig.27, opp:

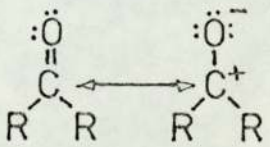


Fig.27:

These studies showed that an acidic environment is essential when fluorescence measurements are made with the oestrogens, whether conjugated or free, in

Oestrogen Assay

Take aliquot of urine (10 ml).

Wash three times with diethyl ether (3x10 ml).

Discard organic phases.

Adjust pH of washed urine to pH 6-7.

Allow urine to equilibrate at 37°.

Add B-glucuronidase preparation (30 units).

Incubate at 37° for 15 min.

Cool hydrolysate on cracked ice.

Extract with diethyl ether (10 ml).

Wash ethereal phase with sodium carbonate solution at pH 10.5 (10 ml).

Transfer an aliquot of the organic phase to a stoppered fluorescence cuvette and record the fluorescence emission intensity at 310 nm with an exciting radiation of wavelength 284 nm.

Compare the emission intensity with that of a standard solution of oestradiol in diethyl ether.

Fig.28:

Method for the assay of conjugated urinary oestrogens by native fluorescence.

(Solvent method).

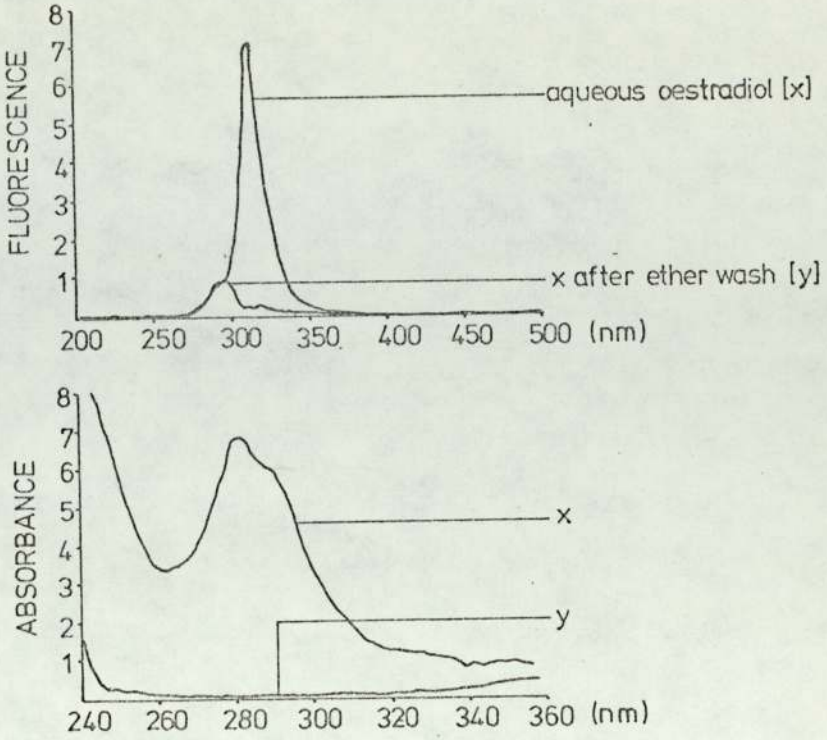



Fig.29: Aqueous oestradiol solution
Effect of ether wash.

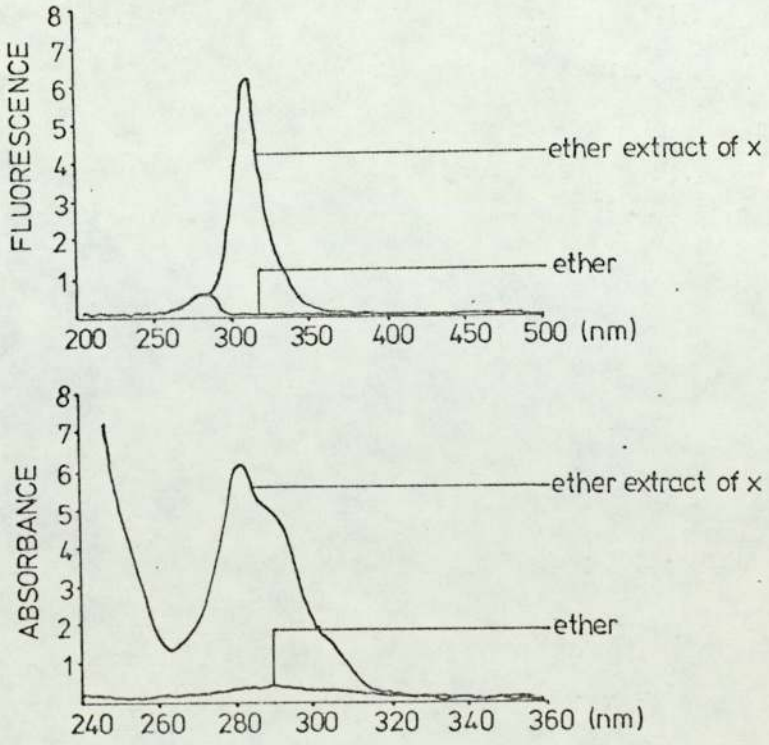



Fig.29: Ether extract of aqueous oestradiol solution; (compared with pure ether)

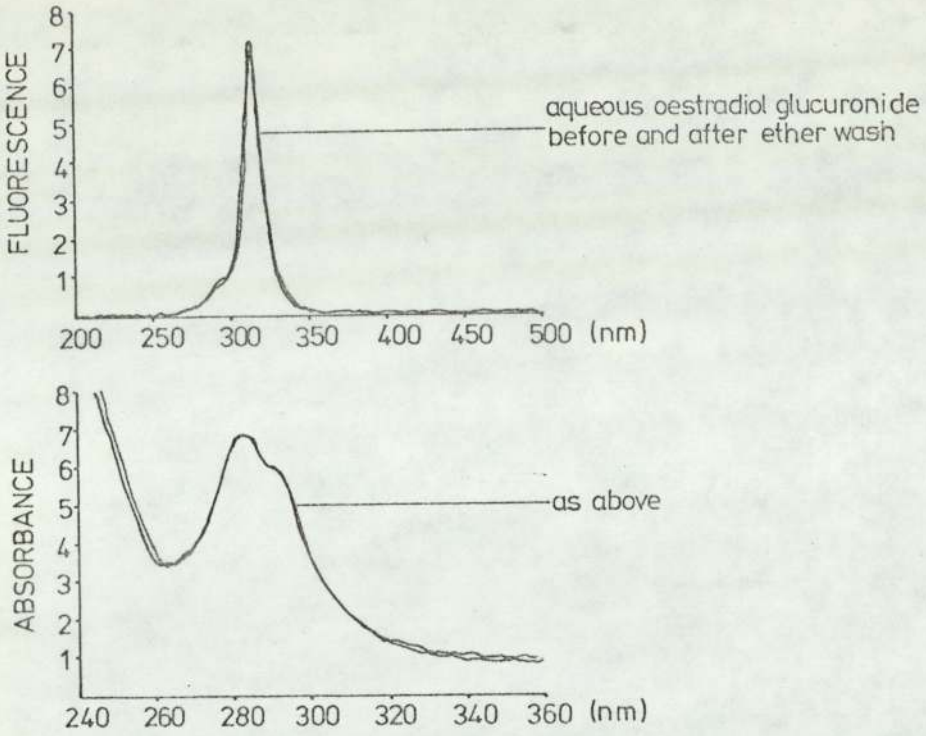



Fig.30: Aqueous oestradiol glucuronide solution Effect of ether wash.

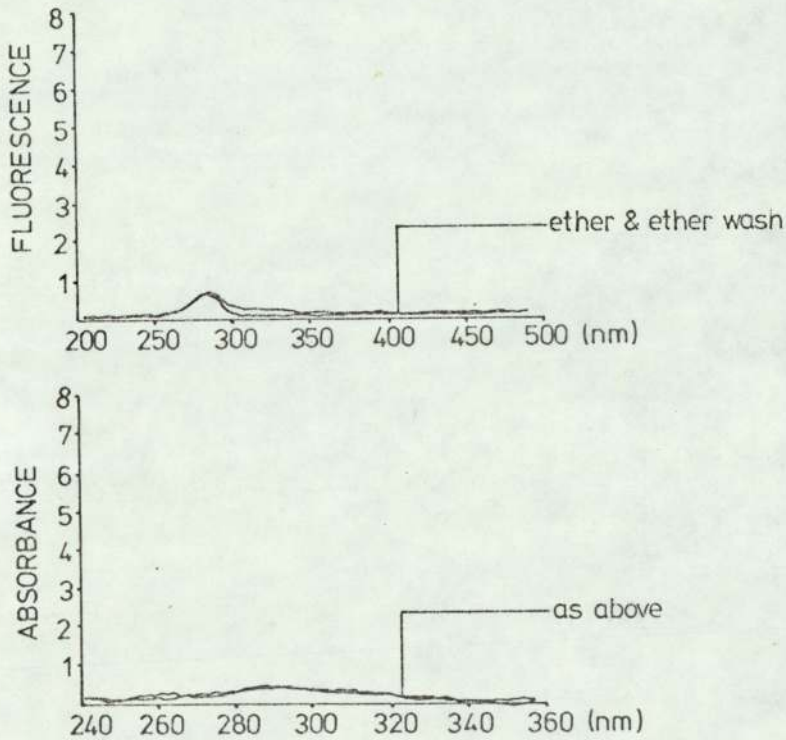



Fig.30: Ether extract of aqueous oestradiol glucur-onide solution; (compared with pure ether)

aqueous solution. The paradox exists, though, that the oestrogens are relatively insoluble in acidic water, and, as a consequence, it is preferable to extract the oestrogens into an organic phase and study their emission in the organic solvent. However, the conjug--ated oestrogens, which constitute the major proportion of the urinary oestrogens, are quite insoluble in organic solvents. In order to extract the oestrogens from urine into an organic phase, the conjugated steroids must first be freed by hydrolysing the gluc--uronic acid residues at their point of juncture with the steroid. The free oestrogens are then capable of being extracted into an organic solvent such as ether (in which the oestrogens display essentially the same absorption and fluorescence characteristics as in ethanol).

The oestrogen assay described in Fig.28 has been developed as a result of a large number of exper--iments designed to achieve the most rapid assay procedure compatible with accuracy and reliability. The fluorescence characteristics of oestrogens are the same in ether as they are in ethanol, and diethyl ether has the vital advantage of being largely immiscible with water.

That the oestradiol dissolved in water can be extracted with very high efficiency into ether is shown by Fig.29, and Fig.30 shows that the conjugated oestrogens found in urine (typified by oestradiol glucuronide) are not extracted to any great extent

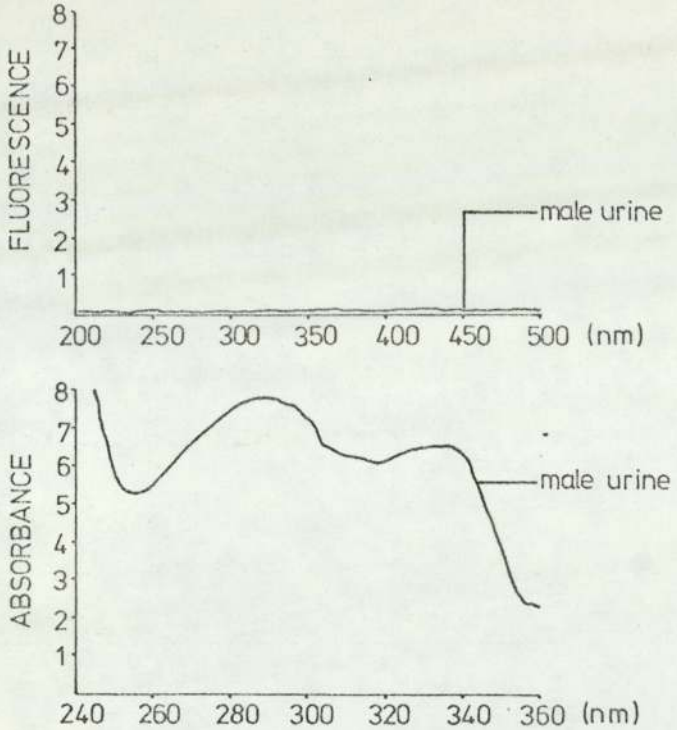



Fig.31: Ether extract of male urine, to show intense absorption of fluid.

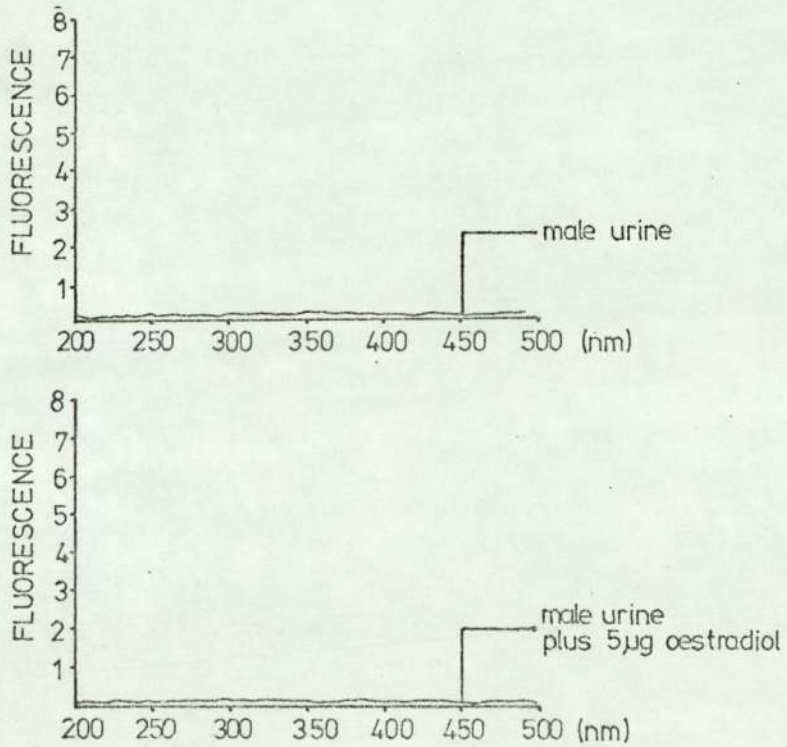



Fig.32: Ether extract of male urine, with and without added oestradiol-17B.

from water into an ether phase. So, by washing the urine with ether it is possible to remove all the ether-soluble components originally present in the urine, yet still retain the oestrogen conjugates. There are a very large number of ether-soluble components present in urine, many of these are phenolic, and the ether extract of a male urine sample, as Fig.31 shows, is intensely absorbing. (Any fluorescent components, of which there are many, are not evident because of the self-absorption of the solution). If oestradiol is added to male urine before the ether wash it remains quite undetectable by native fluorescence or absorbance determinations because of the presence in the urine of these contaminating chromophores (Fig.32). These can be removed by extract--ing the urine with ether, but, in doing this, there is the unavoidable loss of those oestrogens which were present in an unconjugated form. Since approximately 95% of urinary oestrogen is conjugated with glucuronic acid[78] it is clear that the conjugated oestrogen determinations are an extremely close approximation to total oestrogen determinations, and the test cannot be criticised on this basis.

Having washed the urine free of ether-soluble components, the urine is then warmed to 37° in a water bath, and kept at this temperature for 2-3 min prior to the addition of enzyme. The preparation used was a B-glucuronidase of high specific activity obtained

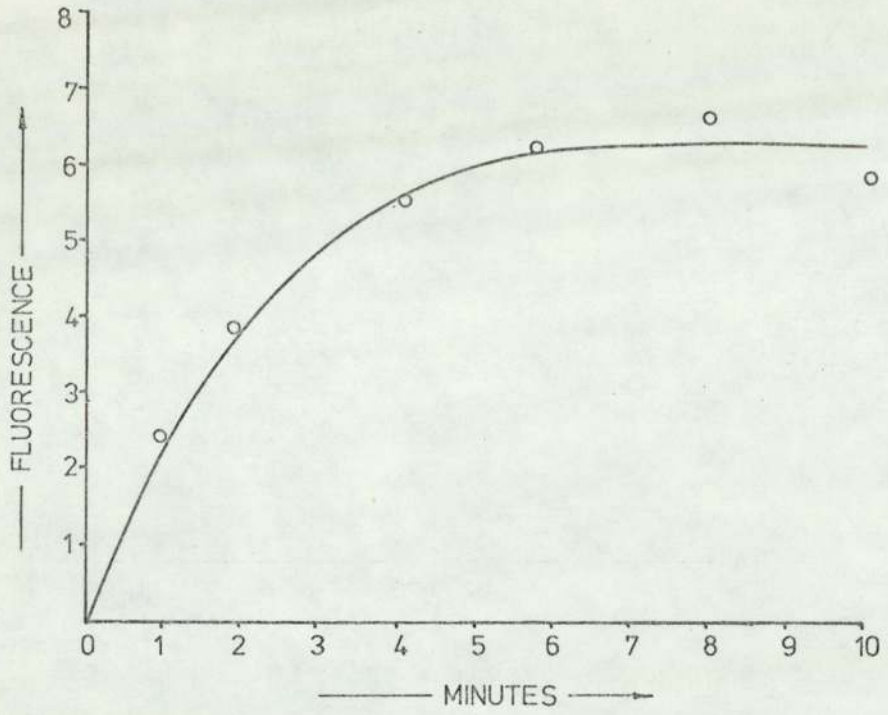



Fig.33:

Rate of liberation of free oestradiol when $^{14}C$-labelled oestradiol-17-glucuronide is subjected to 3.0 Units of B-glucuronidase at 37°. Reaction parameter being the emission intensity of ether extracts of the incubation mixture taken at time intervals shown above. Excitation at 284 nm, and emission at 310 nm.

from a strain of E. coli. The enzyme was reported in 1972[106] and is commercially available. The recommended incubation period is 2 hours at 37° at an enzyme concentration of 1.0 Unit per ml of urine. Crowley[82] has established that a concentration of 3.0 Units per ml allows complete hydrolysis of the oestrogen conjugates in 15 min at 37°, and this method of accelerated hydrolysis has been adopted in the assays described here. Fig.33, opposite, confirms Crowley's findings, and shows that, in the current work, hydro--lysis was complete after 7 min. It is preferable to use a shaking water-bath during the hydrolysis, since this ensures complete mixing of the mixture, and an even temperature distribution, but static tubes, if they are swirled once or twice during the hydrolysis, offer a suitable alternative.

After hydrolysis, the urine mixture was cooled to 4-5° on a bed of ice. At this stage the urine contains no ether soluble components except those which have been formed during the course of hyrolysis. Unlike an acid hydrolysis, the enzyme preparation is extremely specific in its attack, and it is clear that the only reactions which have occurred will be those involving the cleavage of glucuronic acid residues from other organic moieties. The molecules of principal interest are the oestrogens, and the previous conjugated (and ether insoluble) oestrogens will now be present as free (ether-soluble) steroids; so an extraction of the mixture, at this stage, with

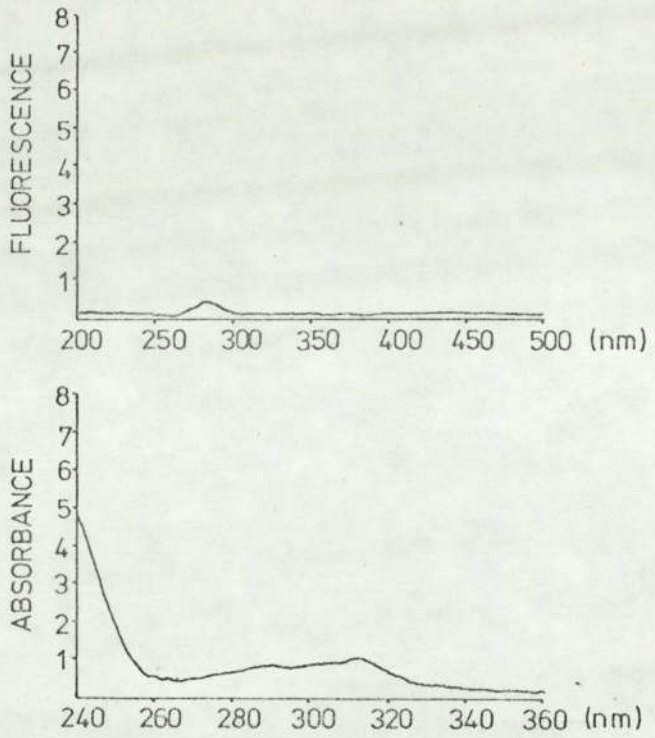



Fig.34: A sample of male urine subjected to the assay for oestrogens as described in Fig.28. There is no extracted fluorescent compound, and the absorption is relatively low.

diethyl ether will carry with it these newly liberated free steroids. Naturally, the oestrogens are not the only molecules which will have been liberated from conjugation by the enzyme. Many other substances are present in the urine in a conjugated form, and these too will have been rendered ether-soluble by the procedure. It is here that the specificity of the fluorescence assay is of most value. Alone amongst the steroids, oestrogens possess an aromatic centre, and it is this phenolic chromophore which gives the molecules both their biological activity and their fluorescence properties. Thus, it is of no consequence that other steroids are liberated, and are free to be extracted into an ether phase, because they will not exhibit native fluorescence, and hence will remain undetected. Trials showed, too, that these liberated compounds did not exert any significant quenching effects which would interfere with the oestrogen assay. Fig.34 shows that male urine, when subjected to the procedure described in Fig.28 showed no native fluorescence (indicating no release of fluorescent components, such as conjugated aromatic compounds, during the hydrolysis procedure), and Fig.35 shows that oestradiol glucuronide could be extracted and assayed in male urine with the same efficiency and accuracy as from water.
Table 3 (overleaf) shows that the assay procedure extracts approximately 95% of the oestrogen originally present in the urine. The greatest loss, accounting

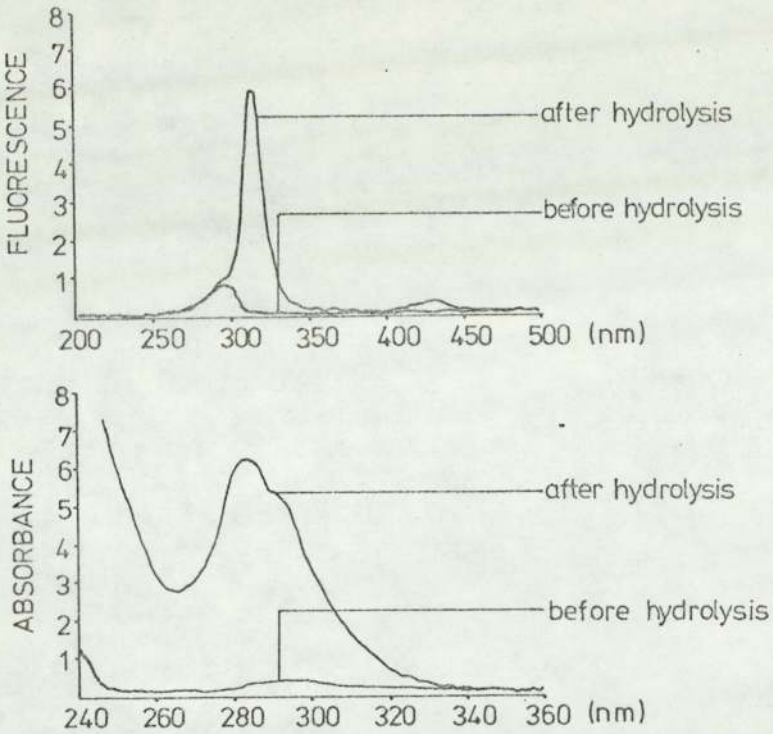



Fig.35: Ether extract of aqueous oestradiol glucu--ronide; before & after enzyme hydrolysis.

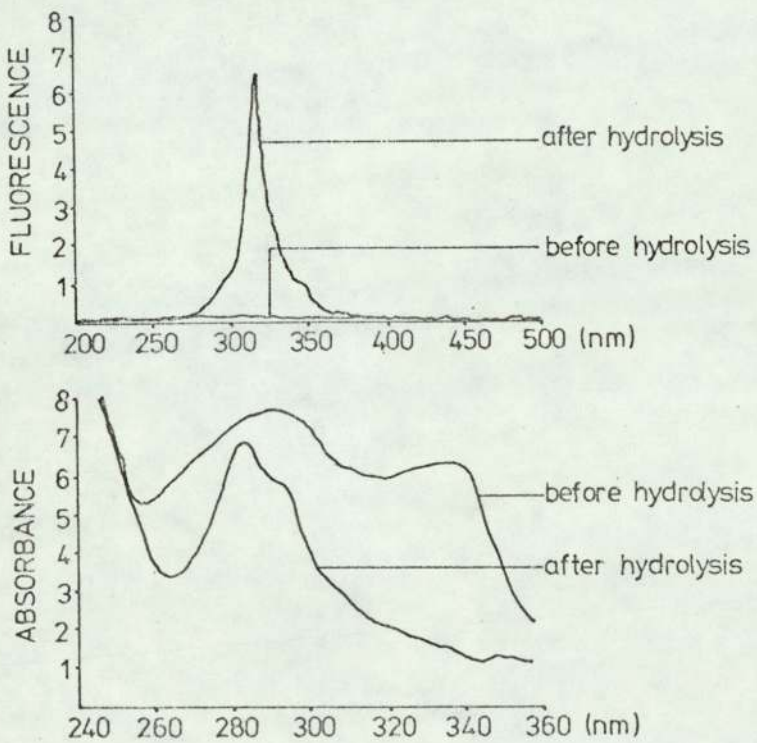



Fig.35: Ether extract of male urine, containing oestradiol glucuronide, before & after enzymic hydrolysis.

| sample | cpm |
|---|---|
| Ether extract of urine | 1,387 |
| Ether extract after hydrolysis | 1,401 |
| Ether extract after hydrolysis & carbonate washing | 1,286 |

Table 3:

Showing the results of processing $^{14}C$-labelled oestradiol through the extraction procedure of the oestrogen assay.

for almost all the oestriol not recovered, occurred in the stage where the ether extract of the hydrolysed urine was washed with sodium carbonate solution. This step is essential, however, since it removes, almost completely, non-oestrogenic compounds which have fluorescence emission characteristics similar to those of the oestrogens. But even more important is the removal of materials which, although non-fluorescent, are present in large quantity in the urine, and which, because of their intense absorption of ultra-violet radiation, would quench the oestrogen fluorescence to an appreciable extent. This effect is readily illustrated by the comparison between the absorption and emission spectra of the ether extract of the urine hydrolysate before and after the carbonate washing stage (Fig.36, overleaf).

It is important that the pH of the carbonate wash solution is exactly 10.5. This must be checked

regularly before use. A higher pH will increase the solubility of the oestrogens because of their acidity as phenols, and they will then be extracted into aqueous medium, resulting in a decrease in the efficiency of extraction of the method. Even at pH 10.5 it is clear from Table 3 that approximately 6% of total oestradiol present in the sample of urine is lost through this washing. The only other sources of oestrogen loss are those which might arise during the manipulation of the samples. Care needs to be exercised in transferring solutions of oestrogens from vessel to vessel, since the polar nature of these steroids may cause them to absorb onto glass surfaces and produce an apparent loss.

The contribution to the 'oestrogen fluorescence' by those non-oestrogenic constituents of the urine not completely removed by the washing procedure was determined by assaying a sample of male urine. It was considered that such constituents would be common to both male and female urines to about the same extent. Fig.34 shows that a male urine showed a very low level of fluorescence emission, and that this level, when compared with that from a standard solution of oestradiol in ether, was found to be equivalent to that which might be expected from a sample of preg--nancy urine showing an excretion rate of 0.28 mg per 24 hours. (Similar experiments were performed with samples of non-pregnent female urine, and the oestrogen equivalent in these cases was approximately 0.30 mg

per 24 hours). Background levels of this order, contributed to by non-oestrogenic urine components, do reduce the absolute accuracy of the method, but are without effect on the diagnostic value of the test.

The reported lower limit of detection of oestradiol by means of its native fluorescence inten--sity is 10 $ng.ml^{-1}$ [433]. This, in terms of a 2 litre 24 hour urine sample, is equivalent to about 20 ul per 24 hours. At first consideration, this is a level of sensitivity almost suitable for the determination of oestrogens in non-pregnancy urine (Fig.15), but in practice this is difficult to achieve with standard equipment. One of the greatest obstacles is the background emission of solvents at the amplifications necessary to measure such low intensities of fluores--cence. It was found that the use of technical grade ether for solvent extraction generally produced high solvent emission values. The impurities which exert this effect could be removed, to a large extent, by distilling the ether from KOH pellets. However, it was found that many batches of 'Analar' grade ether had acceptably low emission backgrounds and could be used as supplied provided they were checked first.

A further obstacle to increasing the sensit--ivity of the method was the closeness of the excit--ation and emission wavelengths which leads to the interference of the latter by the former unless extremely efficient monochromation is achieved. It was

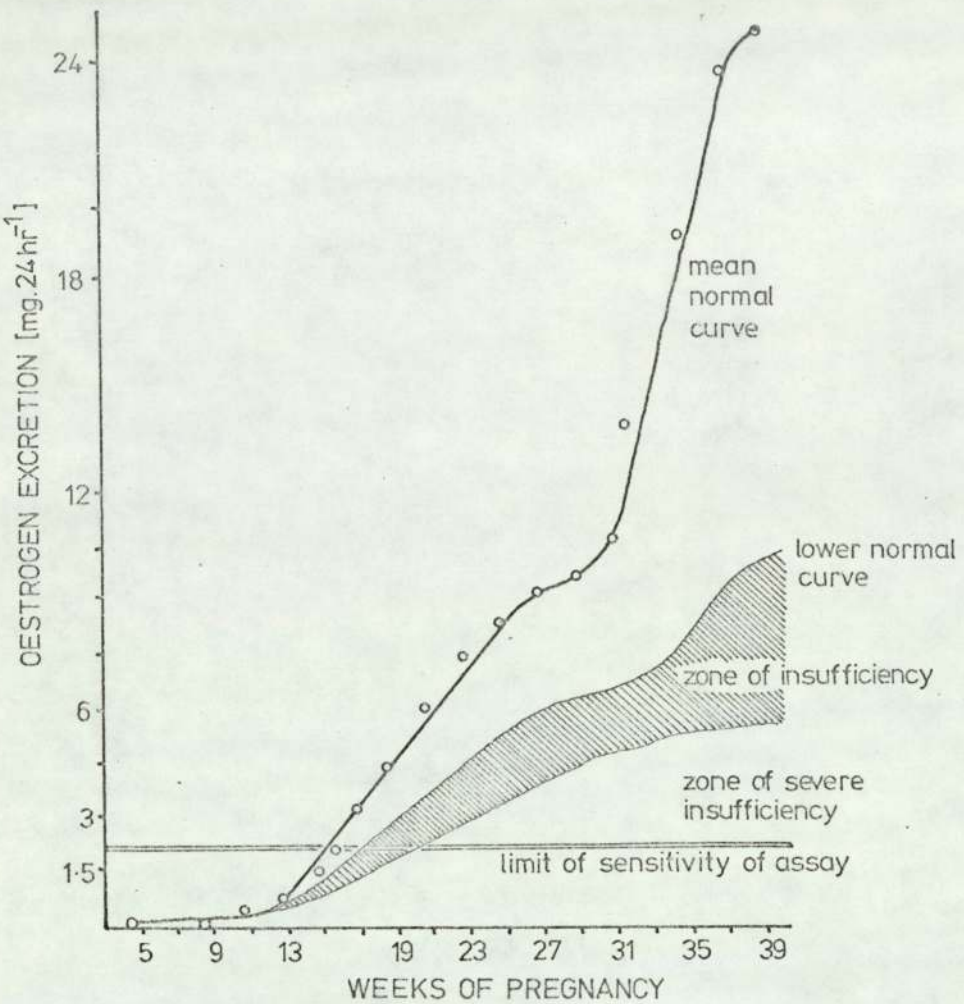



Fig.37:

Mean normal excretion of oestrogens during pregnancy showing zones of feto-placental insufficiency (data from Jayle[178]), with limit of sensitivity of new assay for the urinary oestrogens superimposed.

found, however, that by a judicious selection of instrumental characteristics such as high-resolution, high-brilliance monochromators and high-sensitivity phototubes this difficulty could be minimised. The conditions described in Fig.28 do allow the determin--ation of oestradiol and oestriol in urine at excretion rates of 2.0 mg per 24 hours, or higher, with a great degree of precision (Table 4), and this level of sensitivity is more than adequate for pregnancy urines from the second half of pregnancy, as Fig.37 shows.

| Sample | Oestrogen Conc. (mg/24 h) | |
|---|---|---|
| 1 | 5.8 | 6.5 |
| 2 | 8.5 | 8.7 |
| 3 | 8.7 | 8.8 |
| 4 | 8.9 | 8.7 |
| 5 | 8.5 | 8.6 |
| Mean | 8.17 | |
| Precision | 0.24 | |
| Precision as % mean | 4.79 | |

Table 4:
Duplicate values obtained for five estim--ations of oestrogens in 24 hour urine samples.

Using this assay procedure it has been possible, in this laboratory, to determine the oestrogen concen--tration of a sample of pregnancy urine within 25 min of its receipt. The method is ideally suited for those

Oestrogen Assay

Take aliquot of urine (10 ml).

Add ammonium sulphate (6.5 g).

Shake until fully dissolved.

Centrifuge to sediment precipitate.

Harvest pellet; add water at 37° (10 ml).

Add B-glucuronidase preparation (30 units).

Incubate at 37° for 15 min.

Cool hydrolysate on cracked ice.

Extract with diethyl ether (10 ml).

Wash ethereal phase with sodium carbonate solution at pH 10.5 (10 ml).

Transfer an aliquot of the organic phase to a stoppered fluorescence cuvette and record the fluorescence emission intensity at 310 nm with an exciting radiation of wavelength 284 nm.

Compare the emission intensity with that of a standard solution of oestradiol in diethyl ether.

Fig.38:

Method for the assay of total oestrogens from pregnancy urine by native fluorescence. (Precipitation method).

instances where there are a small number of samples which need to be assayed relatively rapidly, but, because of the manipulation needed for each sample, the method is not as rapid with large numbers (more than 25) of urines, and an alternative procedure was developed for use in busy laboratories. The 'rapid' method is ideally suited to automation on a contin--uous flow basis, but such a development did not fall within the scope of this thesis.

As early as 1936, Cohen & Marrian had demonstrated the precipitation of oestrogen glucuronidates from aqueous solutions with ammonium sulphate[80] . The technique was quantitated more recently[77] , and Fig.38 shows how this technique has been adapted as part of a second rapid assay for oestrogens which is much more applicable to the assay of large batches of urines.

The addition of 65% ammonium sulphate approaches saturation point for most urines. The precipitate contains many substances which are themselves fluor--escent under appropriate conditions, but most of these substances (of which there are a wide variety) appear to be insoluble in ether. Conjugated steroids are extracted with great efficiency, and the efficiency of precipitation increases as the polarity of the steroid involved in the conjugate decreases. Thus, oestrogens are precipitated with greater effic--iency than keto-steroids or corticoids; but, again,

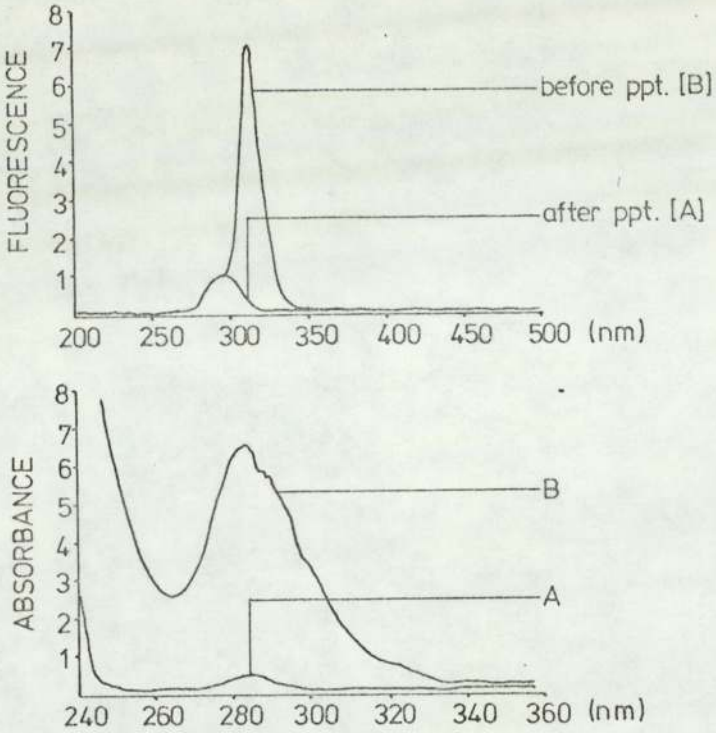



Fig.39: Aqueous oestradiol glucuronide solution
Effect of ammonium sulphate precipitation.

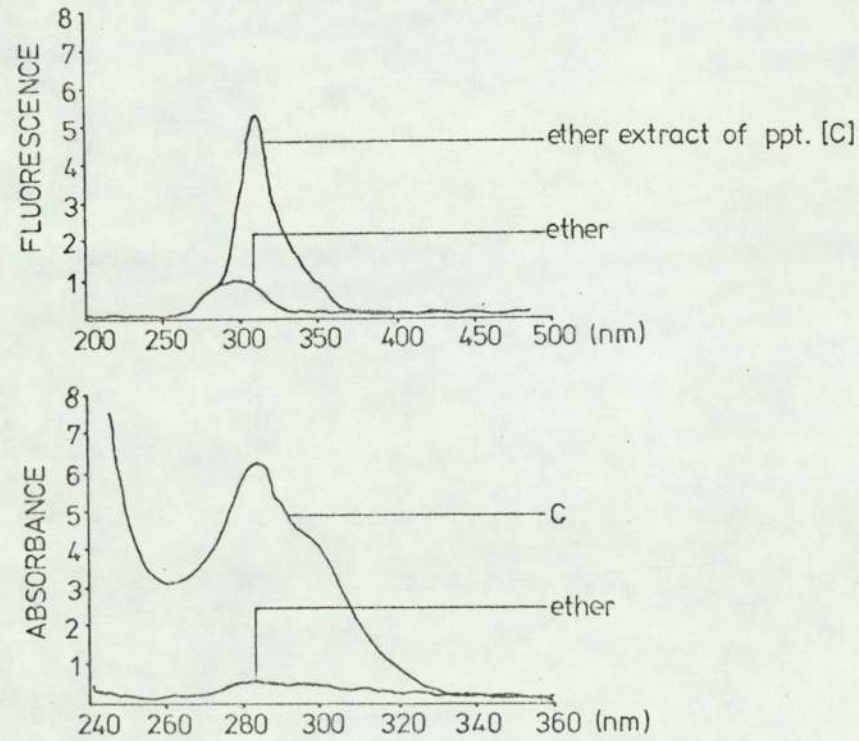



Fig.39: Ether extract of precipitate from
oestradiol glucuronide solution,

oestrogens, alone among the steroids, are the only molecules which are exhibiting native fluorescence. Cohen[77] cites 92% efficiency for the precipitation of urinary oestrogen, whilst Pinkus[310,2] cites 91-104% recoveries for oestrogens from amniotic fluid using a similar system. We have achieved quantitative precipitation of free oestriol and oestradiol, as well as their conjugated derivatives, from control urines, and have noted a remarkably broad range of pH (5.5 to 9.0) over which the formation of the precipitate seems to be effected with equal efficiency. In this laboratory, the ammonium sulphate precipitate has been harvested using an MSE Superspeed centrifuge, spinning at 30,000 rpm for 20 min at 4°; however, it has been established that the precipitate can be harvested with equal efficiency by spinning at much lower speeds with the modern batch swing-out rotors. Such compact bench-top centrifuges are capable of processing 50-100 tubes in one run, which would enable large numbers of oestrogen assays to be completed in minimal time. In the work detailed here, polycarbonate tubes, equipped with aluminium caps, have been used, but caution should be exercised in the choice of materials since steroids are known to be absorbed onto certain acrylic plastics. Fig.39 shows represen-tative spectra obtained with this technique, and comparison with Fig.29 shows that both the methods yield very similar results. Table 5 (overleaf) shows

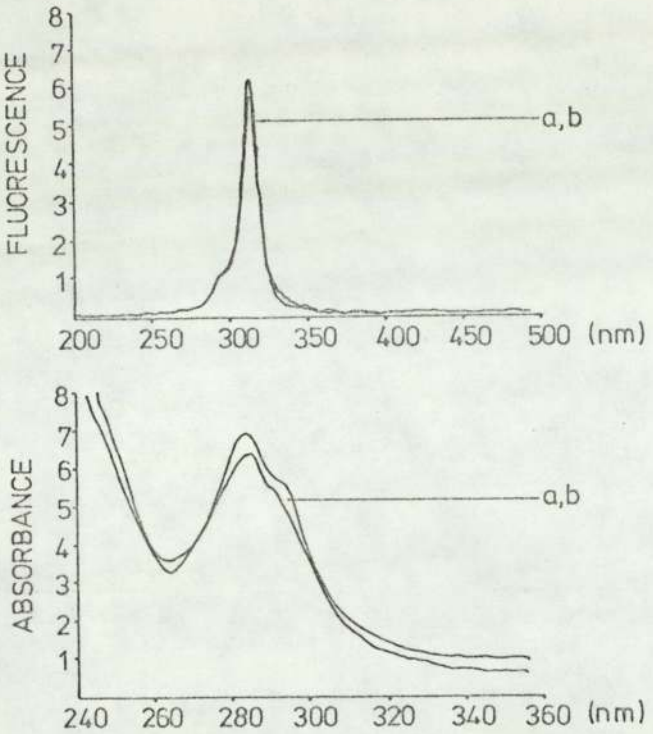



Fig.40: Aqueous oestriol solution; a) before & b) after benzene/petrol washing.

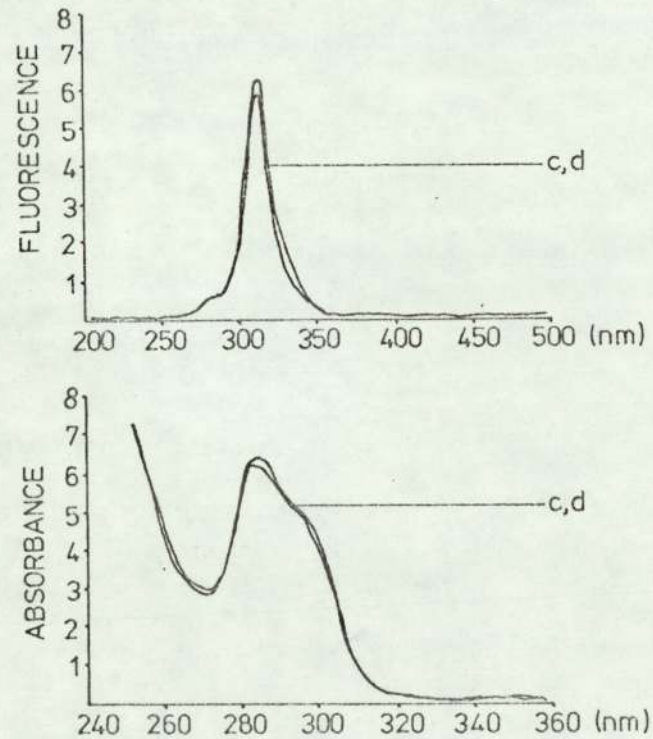



Fig.40: Ether extracts of solutions a (c) and b (d),

the efficiency of retrieval of an internal standard of radioactive oestriol by the precipitation method, and this too compares favourably with that of the rapid method (see Table 3).

| sample | cpm |
|---|---|
| Ether extract of urine | 2,465 |
| Ether extract of ppt. after hydrolysis | 2,394 |
| Ether extract of ppt. after hydrolysis & carbonate wash | 2,262 |

Table 5:
Showing the results of processing $^{14}C$-labelled oestriol through the extraction procedure of the oestrogen assay described in Fig.38.

If, for any clinical reason, it is considered necessary to measure urinary oestriol rather than total oestrogens, then this may be carried out using either of the two methods. The procedures are exactly as described except that before the cooled hydrolysate is extracted with ether it is first washed with a mixture of benzene/light petroleum (b.p. 60-80°) (1:1 v/v). This removes the oestradiol and oestrone present in the hydrolysate and leaves behind the oestriol. The aqueous solution (washed hydrolysate) is then subjected to the ether extraction and all subsequent steps of either of the two described methods. Figs.40 & 41 show the spectra obtained with oestriol assays carried out on dist. water inoccu-

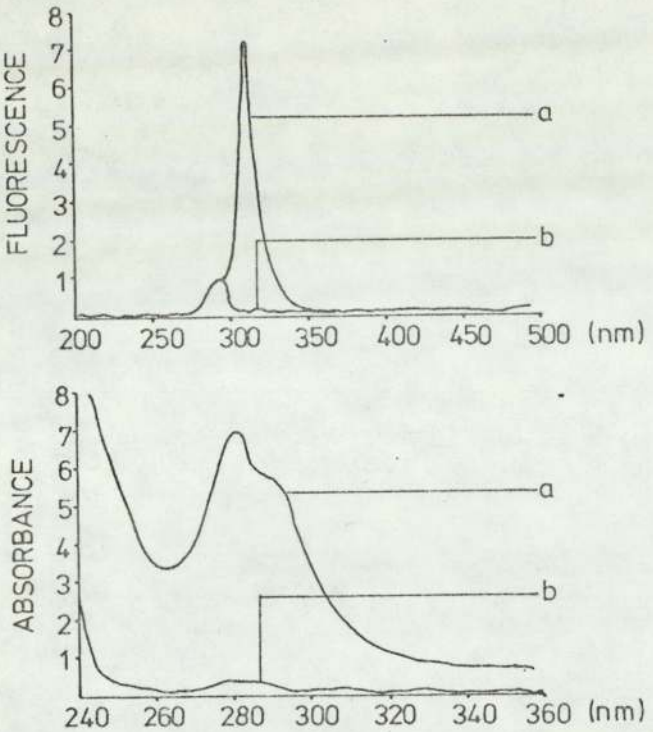



Fig.41: Aqueous oestradiol solution; a) before and b) after benzene/petrol washing.

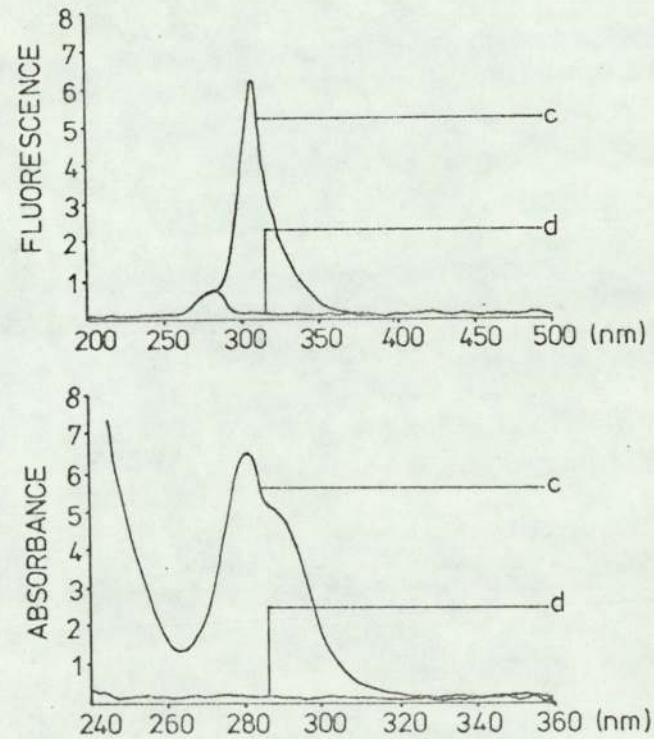



Fig.41: Ether extracts of solutions a (c) and b (d).

-lated with oestriol. These spectra are typical of those obtained throughout the development of this work. As noted before, the equipment used for the fluorescence studies has been the standard Aminco-Bowman apparatus. It was found useful to record spectra on an x-y recorder, but a very large number of trials has shown that a fixed wavelength excit--ation (284 nm) and emission (310 nm) instrument is suitable for all assays. The methods are robust enough to be applied in conjunction with almost any reasonably accurate fluorimeter, even instruments operating on a filter basis, rather than a diffraction monochromator, but the instrument should always be calibrated before assay results are collected. This calibration can be made quite easily with the use of a standard oestriol, or oestradiol, solution.

The traces included with this thesis show that oestriol and oestradiol have very similar fluores-cence and absorbance profiles. Oestrone, as has already been mentioned, has a ramarkably diminished quantum yield of conversion, which is attributable to the ketonic function at position 17 (see page 76). However, it has been shown that no intermolecular quenching occurs between oestrone and oestradiol in a solution containing the two molecular species, and that the amount of oestrone present in late pregnancy urine is proportionally so small as to be neglected. Thus, the fluorescence of the assay extract can be taken to be a direct estimate of the amount of oestrogen in the urine.

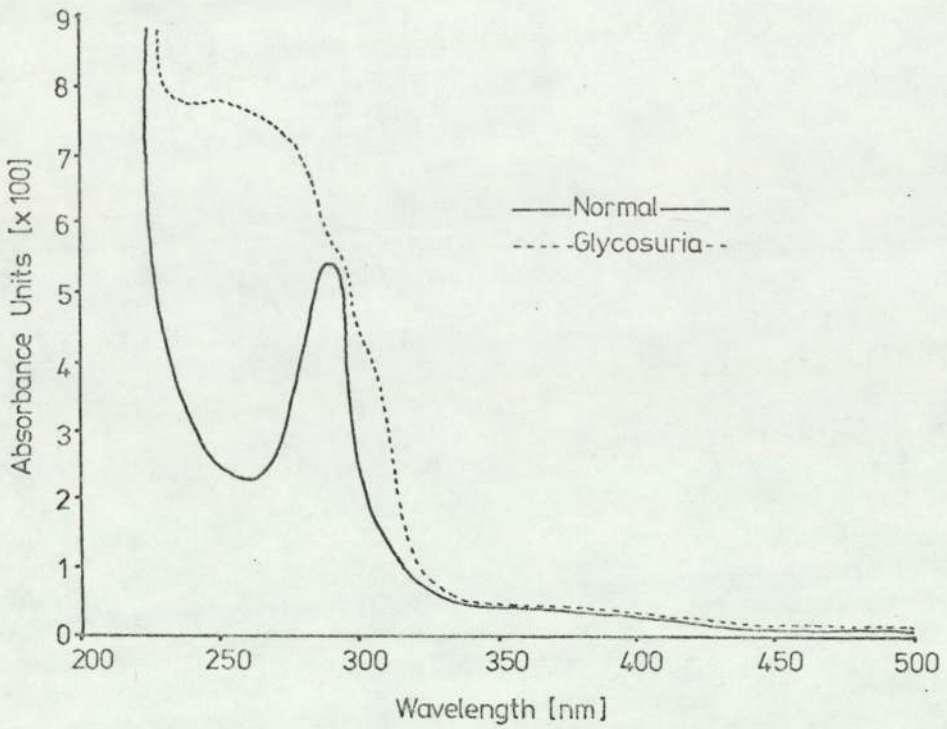



Fig.42:

Absorption spectra of extracts from normal pregnancy urine and urine from a patient exhibiting glycosuria.

It is recommended that an observation of the absorption profile be performed on each solution used for fluorescence assay. Marked idiosyncrasies have been noted in the absorption spectra of samples originating from patients with glycosuria, which might otherwise have gone unrecorded. These abnormal effects may well be a contributory factor in the unacceptably wide variation in results obtained from the previously reported methods of oestrogen determination. The value for the same sample may be as low as 70% below, or up to 200% above a mean 'correct' value, depending upon the laboratory[292]. This does not condemn the methods, since some laboratories obtain consistant results 'close to the correct value'. The conclusion has to be drawn that some of the steps in these methods are very susceptible to operator error, or are dependent on the nature of the sample which is not of consistent constitution. We believe that it is the variation in the latter which produces the major errors. Evidence for this is found in the observation[434] that oestriol is degraded by certain constituents in some urines, and also in my observations of the absorption spectra of the oestrogens extracted from different urines. It was found that out of 48 different samples the extract from 46 showed the expected phenolic chromophore absorption, but in 2 of the samples the extract contained materials other than oestrogens which had not been removed in the extraction process.

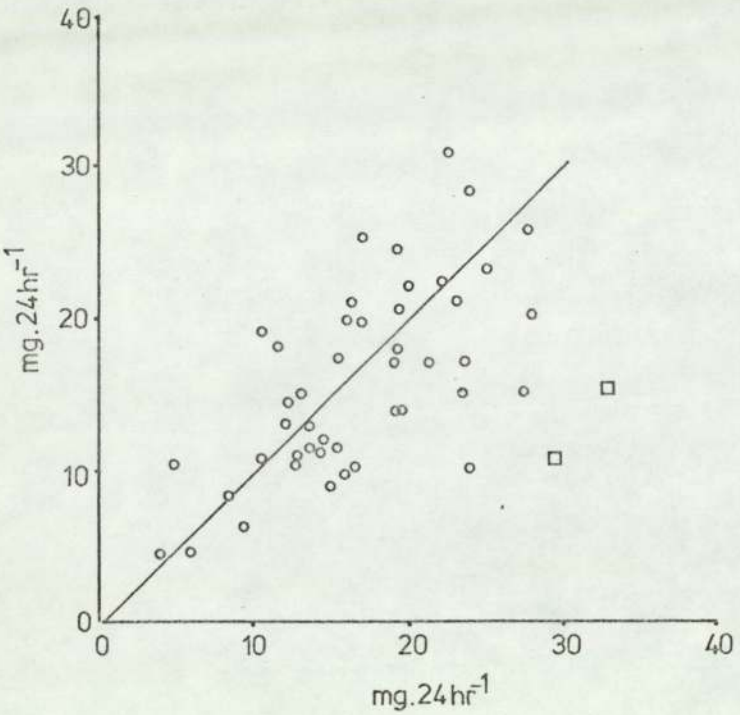



Fig.43:
48 samples of pregnancy urine assayed for oestrogen content by native fluorescence assay (ordinate), and by a modified Oakey method (abscissa).

The absorption patterns of 'normal' and 'abnormal' oestrogen extracts are shown in Fig.42. The two samples with the abnormal absorption when assayed for oestrogens by a modified Oakey method appeared to contain levels of oestrogens considerably higher than the mean normal value associated with the stage of pregnancy of the subjects. Fig.43 shows the correlation plot for these 48 samples, assayed by both the native fluorescence method, described here, and by the modified Oakey method, in the laboratories of the Maternity Hospital which provided the samples. The two data points marked □ are those from the patients exhibiting glycosuria, and it is to be noted that these two cases represent the extreme high values of the Oakey assay, whilst being median with the native fluorescence method.

When the oestrogen content of these samples were determined by the method described in Fig.38 they were found to be 50-70% lower, and closer to the expected values. Both these subjects were discovered to be exhibiting glycosuria. It appears, therefore, that some urine samples contain substances which interfere with the Oakey method for oestrogens and this interference is clearly out of operator control. (It is noted that the Brombacher method[57] is more likely to be free of this interference). It has been suggested that the degradation of oestriol by these urine constituents occurs during the acid hydrolysis of the oestrogen conjugates[293], and may

be largely avoided by the prior precipitation of the conjugates from the urine sample. This is in agreement with the determinations made using the method described in Fig.38, which includes such a procedure and also avoids the use of strong acid in the hydrolysis step.

The methods described in Figs.28 and 38 are likely to be just as applicable to amniotic fluid oestrogen determinations as they are to urine. However, there is no experimental evidence on this aspect, and there appears to be little clinical need for liquor oestrogen assays. As mentioned earlier, the technique of amniocentesis is not yet recognised as routine, and is reserved for the gathering of more specific information about the condition of the fetus than that provided by oestrogen determinations. Plasma oestrogen assays are, however, highly popular with clinicians, and the methods previously described were applied to plasma samples in order to see whether they could be of use in this sector.

For the purposes of this work it was assumed that the concentration of oestradiol present in the plasma in late pregnancy is 20 $ng.ml^{-1}$, and standard solutions of oestradiol were made in both male and non-pregnant female plasma, for use as experimental samples. It was found that neither of the two methods previously described were sensitive enough to detect the oestradiol present in the samples. The problem lay primarily in the signal/noise ratio which was

too small for acceptable determinations to be made using the standard Aminco apparatus.

When a sample of oestradiol-4-$^{14}$C was used as an internal standard in the plasma samples it was clear that the oestradiol was being retrieved by the methods with great efficiency; but, at the high signal amplifications which would be neccessary to determine the low levels of oestradiol present, and with the fluorimeter adjusted for maximal sensitivity (wide slit settings), the contamination of the signal with scattered source radiation became intolerably high. Attempts to reduce the instrumental amplification required by using reflective cuvettes, only enhanced the noise, and attempts to reduce this contamination by placing absorption filters between the sample and the emission monochromators; and also by using a $Mg^{++}$hollow cathode lamp as an exciting source, all produced little benefit. It was concluded that, if a plasma assay was to be developed, it would need to be highly specific in the extraction stages, in order to reduce the concentration of contaminants in the fluorescence solution which would otherwise contribute to background noise.

A number of existing extraction procedures, described for use with plasma, were tried and found to be unsuitable for use with the fluorescence assay (mainly because of problems with the background noise). However, a method based on the collection of oestradiol by SHBG-binding[301] showed more promise.

Oestrogen Assay

Take aliquot of plasma (4 ml).

Add 'dilute ammonium sulphate' (36 ml).

Stand mixture for 10 min at 4°.

Centrifuge to sediment precipitate.

Harvest pellet; add water (3 ml).

Add 'concentrated ammonium sulphate (3 ml).

Stand mixture for 10 min at 4°.

Centrifuge to sediment precipitate.

Harvest pellet; add water (1 ml).

Extract aqueous solution of pellet with benzene/ether (2:3 v/v) (2x5 ml).

Evaporate extract to dryness under nitrogen.

Take up residue in ethanol (3 ml).

Transfer alcoholic solution to a stoppered fluorescence cuvette and record the fluorescence emission intensity at 310 nm with an exciting radiation of wavelength 284 nm.

Compare the emission intensity with that of a standard solution of oestradiol in diethyl ether.

('Dilute': Saturated (4°) $(NH_4)_2SO_4$:water (10:9))
('Concentrated': Saturated (4°) $(NH_4)_2SO_4$)

Fig.44:

Method for the assay of plasma oestradiol

by native fluorescence.

Using a RIA assay with this extraction procedure is claimed to give a sensitivity to a plasma oestradiol concentration of 30 $pg.ml^{-1}$, with an overall recovery of 50%[301]. A modification of this basic method is described in Fig.44. Using an internal standard of oestradiol-4-$^{14}C$ it was found that, in this laboratory the overall efficiency of recovery of oestradiol was 68%. The method is based upon the selective precipitation of the globulin fraction of the plasma, which includes the SHBG protein. Since most of the oestradiol present in the plasma is found in this SHBG, a precipitation of the SHBG fraction brings with it the oestradiol present in the sample of blood. The extraction procedure is highly specific for steroids, and, since only the oestrogenic steroids are fluorescent, if the procedure is used in conjunction with a native fluorescence assay, then the overall method is highly specific for oestrogenic steroids.

The original method involved RIA as the assay technique, and this gave the required level of sensitivity. Using native fluorescence, however, it was not possible to reproduce this sensitivity using the standard Aminco fluorimeter. It was established, however, that the final ethanolic extracts from both male and female plasma samples showed a sufficiently low background emission for a determination of oestradiol to be made, provided suitable instrumentation was available. In general, with the Aminco fluorimeter,

no signal can be considered to be significant until it exceeds the mean amplitude of the background emission by five-fold. This backgound noise is contributed to by lamp output fluctuations, amplifier and photomultiplier instability, and solvent effects. These different sources provide signals of differing periodicities, and it is possible, by adjusting instrumental parameters correctly, to decrease this noise to very low levels. Using non-standard fluorescence equipment involving gated signal filters, delay loops and a signal averaging system it is possible to accept a signal as significant when it is only 0.1 of the amplitude of the background emission. Thus, with appropriately designed fluorescence apparatus it should be possible to obtain a level of sensitivity at least 20 times (and possibly two orders) higher than that possible with the Aminco. It is clear, therefore, that the plasma oestrogen assay described in Fig.44 will provide an accurate, sensitive and highly specific method for the determination of plasma oestrogens in early pregnancy, (and even in male plasma) if it is used in conjunction with appropriate detection apparatus. Work currently being undertaken in this laboratory should provide clear evidence of the value of this assay, and should show how signal averaging fluorimeters can extend the sensitivity of existing techniques. However, even when this aspect has been investigated, it is unlikely that the fluorescence assay for plasma

ostrogens will take the place of RIA in many hospital laboratories, and the assay is more likely to remain (in marked contrast to the urinary oestrogen assay) of academic interest rather than practical relevance.

It can be concluded then, that the practical assays described in this section can be used to determine the levels of oestrogens in pregnancy urine, in amniotic fluid and (potentially) in pregnancy plasma. The first of these assays has the most practical relevance and offers very many advantages over those currently available, particularly the widely used spectrophotometric assays.

The basis of the other spectrophotometric methods of oestrogen determination is an acid hydrolysis of oestrogen glucuronides and an acid catalysed chemical reaction to produce the chromagen or fluorphor with the addition of some hydroqinone and nitrophenol reagents in the latter step. The acid hydrolysis has for many years been preferred to an enzymic method owing to its greater rapidity. Using enzyme preparations in the past could take up to 18 hours for complete hydrolysis[82]. It still remains true however that the acid hydrolysis procedure is one that, if possible, would be well to avoid. The conditions are without doubt, in chemical terms, very vigorous. The hydrolysis is invariably carried out at 100° for periods up to 1 hour in 15-80% acid solutions. Under such conditions many an organic molecule succumbs and yields such a variety

of products as to daunt most serious minded chemists. It is well known, for example, that alcohol or hydroxyl group containing molecules, such as oestriol and oestradiol undergo dehydration[47]. This proceeds through a carbonium ion, a highly reactive intermediate, which frequently undergoes rearrangements[194] and polymerisation reactions leading to several products. Steroidal compounds are no exception to such reactions. For example the treatment of the methyl ether of oestradiol with 78% sulphuric acid at 100° for 20 minutes produced sulpher dioxide, hydrogen sulphide and a mixture of dark red resinous products. From the mixture no less than six different compounds were isolated[194].

The use of the enzyme preparation from E. coli avoids these hazards and produces a clean hydrolysate within 15 minutes at 37°. This is clearly the ideal method of hydrolysis and its adoption in the assay described in this thesis is completely validated by the level of accuracy and precision attained as shown in Table 4. Complete hydrolysis within 15 minutes required 3.0 I.U of enzyme preparation. If cost is important then the reduction to 1.0 I.U of enzyme is possible and increases the time of hydrolysis to 30-60 minutes[82]. This hydrolysis procedure is superior to the acid one which should be avoide or very carefully standardised with respect to time, temperature and reagents if accurate results are to,be obtained[47]. Similar observations

apply to the use of acid for the development of the Kober chromagen. The loss of oestriol may be as high as 20% though a combination of acid and urine constituent degradation reactions[57] . A further potential source of error lies in the development of the Kober chromagen. This reaction requires the introduction of hydroquinone or nitrophenol. Unless these reagents are always obtained in a high state of purity and maintained this way throughout their useful life, then they can act as a source of interference in the assays. Neither of these compounds can be attributed with any special stability and if by chance laboratory grades are used then the likelihood of the presence of impurities is high. In the method described in this thesis the introduction of 'foreign' substances and any accompanying contaminants is completely avoided as is also the use of any, let alone vigorous, chemical reactions for the determination of oestrogens. possible source of error in the described method is the use of ether as the solvent for the analytical solutions. It is very volatile and so evaporation losses can be incurred through careless handling. This is, however, very readily rectified by adopting a good manipulative technique, which also includes a check on the ether for its fluorescence content prior to its use and irrespective of the quality of ether obtained.

It can be concluded that the method described in this thesis is outstanding for its performance

and accuracy. It is readily adaptable to an automated analysis of large numbers of urine samples for oestrogen content and should result in a large saving of operator time which will compensate for any cost increase over other methods.

Three modes of action of oestrogen were described in section IV, but only two were discussed. The effects of oestrogens on cells other than target tissue cells must clearly be independent of a receptor-mediated process, and it is tempting to think of cell-respon--siveness to oestrogens being mediated by the cell membranes rather than by interference of intermediary metabolism.

It was clear that if a system could be established in which oestrogens showed a physiological effect which was totally independent of transcriptional control mechanisms, then this system might well provide some insight into the existence of any membrane effects of oestrogens.

Initially, it was decided to use the blood platelet as such a model, for the reasons described below, but it soon became clear that very much more information could be harvested using the lymphocyte as a test cell.

The platelet had been attractive for a number of reasons. Firstly, it is a cell remnant, rather than a true cell; and - lacking any nucleus - it is incapable of exerting any transcriptional control over its metabolism. As a consequence, the scheme of events shown in Fig.7 can not apply to the platelet.

Although I have carried out a great deal of work on the interactions between calcium, oestradiol and the platelet membrane involved in the platelet release and aggregation reactions, this thesis will be concerned only with those results obtained with the thymocyte system (although experimental details applicable to platelets are included for the benefit of future work).

The reasons for this ommision are twofold. Firstly, the information available on the platelet interactions is not yet at a sufficiently advanced stage to allow general considerations to be made about the mode of action of oestrogens; and secondly, the platelet is too specialised a cell type to provide results which could be considered as representative of the bulk of somatic cells within the body. The lymphocyte is a very much better model in this respect, and some considerations of its cell membrane structure and behaviour are given in the forthcoming sections.

...but there is yet another consideration which is more philosophical and architectonic in character; namely to grasp the idea of the whole correctly and thence to view all parts in their mutual relations.

Kant (Critique of Practical Reason)

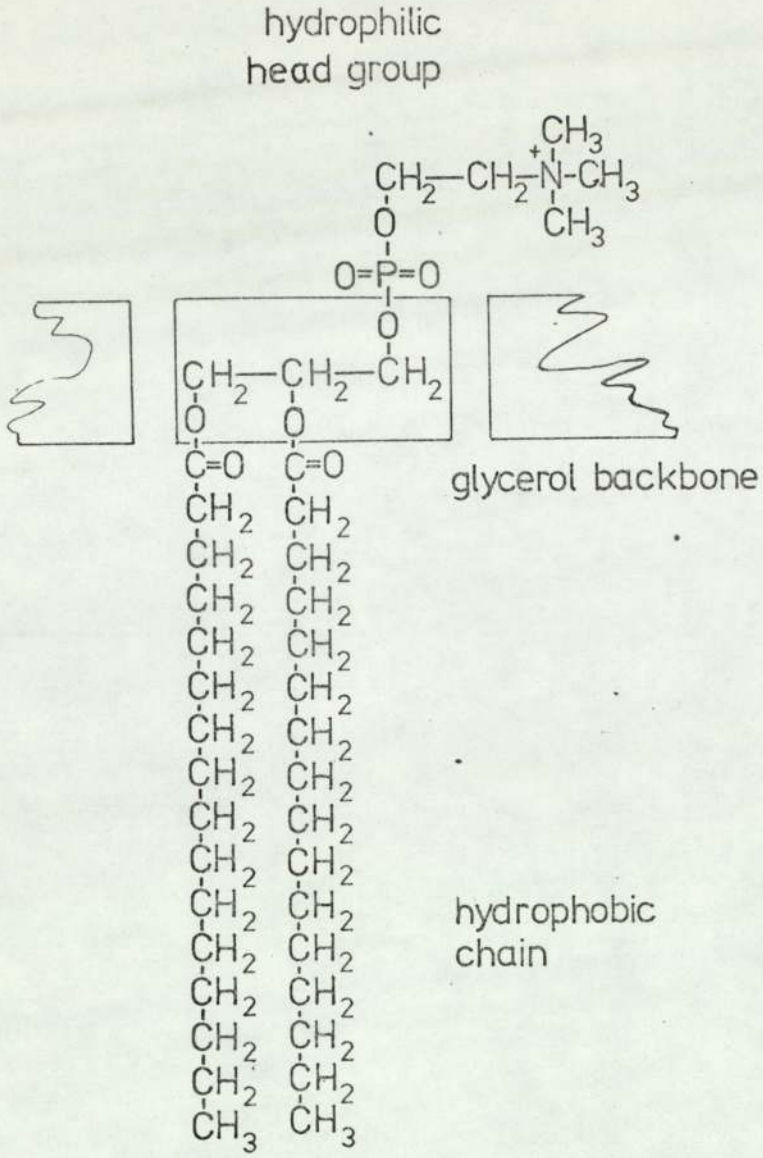



Fig. 45:
A polar lipid based on a glycerol backbone.

## XI Membrane Structure

The essential barrier at the cell surface is the plasma membrane (also known as the cytoplasmic membrane). This may exist alone or may be part of a more complex cell-surface structure. Within cells, the barriers delineating compartments are also mem--branes, their quantity and complexity depending on the type of cell. Membranes, both at the cell surface and within the cell, are essentially lipoprotein structures. The lipids most intimately involved with biological membranes are phospholipids, and these, on their own, can adopt a variety of structures in the presence of water, characterised by a long-range organisation periodic in one, two or three dimensions[241]. The commonest of these structures for the phosphat--idyl cholines (Fig.45) is the lamellar phase which consists of bimolecular leaflets of lipid lying with their planes parallel and separated by regions of water (Fig.46 below):

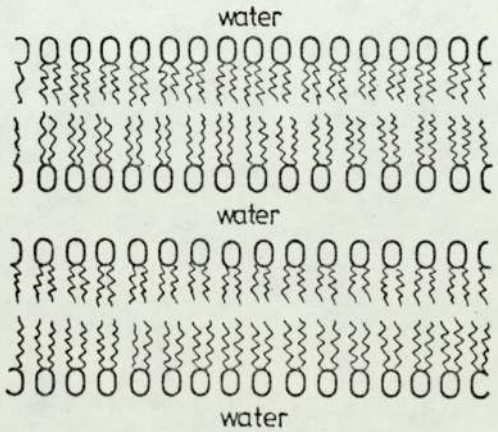



Fig.46:

The lamellar phase adopted by aqueous dispersions of phospholipids.

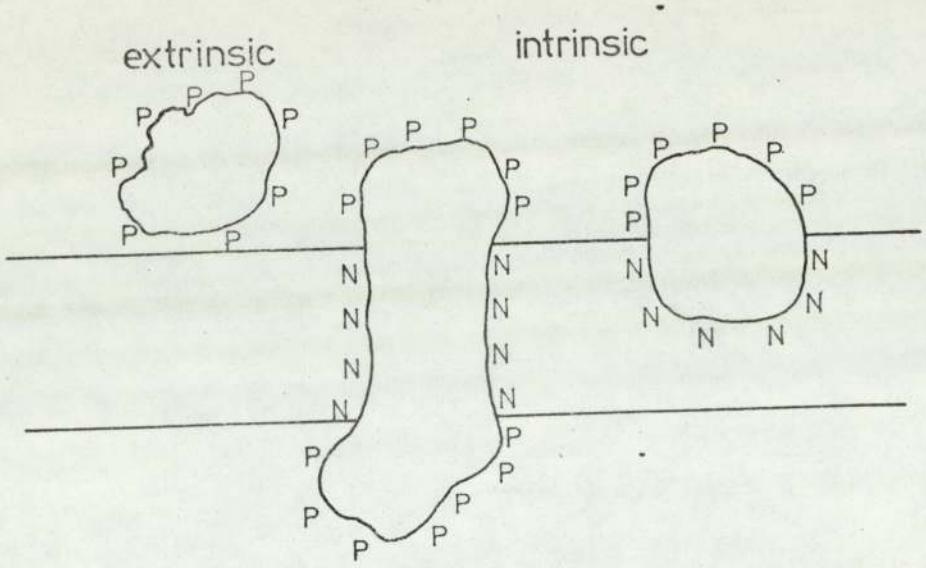



Fig.47:
Location of extrinsic and intrinsic proteins relative to the lipid bilayer of the membrane. The distribution of polar (P) and non-polar (N)-amino acids is shown schematically.

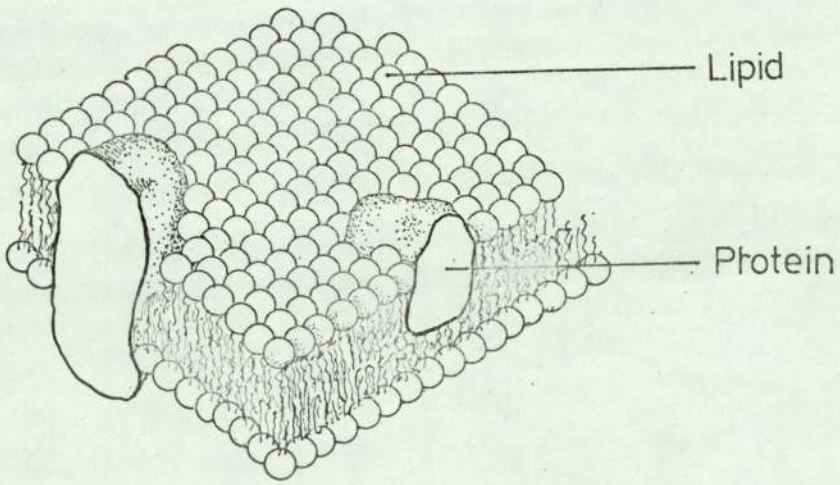



Fig. 48: Fluid mosaic model of membrane structure.

Other, more complex, phases may form in water, but these tend to occur only under conditions of high temperature and low water concentration. However, transitions from one phase to another can be caused by the addition of metal ions. For example, the addition of calcium to an aqueous dispersion of cardiolipin causes a phase transition from a lamellar to a hexagonal phase[326], and there are numerous other examples which might be cited.

The most commonly accepted picture of the biological membrane is of a lipid bilayer with some protein molecules partially embedded in the lipid matrix, and some completely penetrating the bilayer (Fig.47) as first proposed in the 'fluid mosaic' model of Singer and Nicolson[383] (Fig.48). These mem--brane proteins have been classified in two general categories, viz. 'extrinsic' and 'intrinsic', based on their ease of dissociation from the membrane[154]. Extrinsic proteins, also referred to as peripheral or membrane-associated proteins, are relatively easily dissociated by mild treatment such as exposure of the membrane to media of very low or very high ionic strength, or extremes of pH. These proteins are released in soluble, non-aggregated form, and free from contaminating lipid. Intrinsic (or integral) proteins generally require more dramatic treatment, with reagents such as detergents or chaotropic agents before they can be dissociated from their membranes.

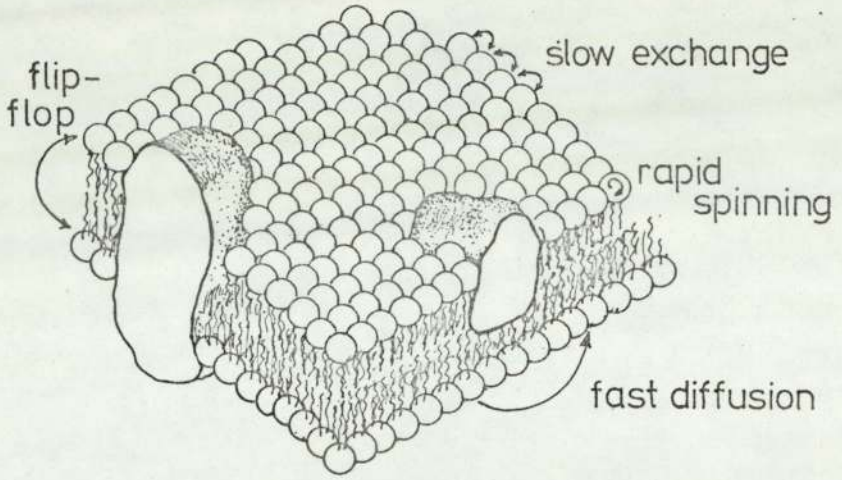



Fig. 49: Possible lipid motions in a membrane.

They often come away with associated lipids, and if these lipids are removed the protein may precipitate or aggregate from solution[154] . Both sorts of protein are believed to be globular, but intrinsic proteins penetrate, and occasionally cross, the hydrophobic interior of the membrane, and thus are often found to have a relatively high proportion of non-polar amino acids in their primary sequences (Fig.47).

The lipid bilayer is not a static structure. There is a continual harmony of motions within the structure[219], and there are several reasons for this activity. Firstly there is the rapid internal motion within each lipid molecule; secondly lipid molecules can diffuse laterally in the plane of the bilayer, (although the rates of these motions could be very different for those lipids associated with proteins and those lipids which make up the bulk of the mem--brane). Thirdly, lipid molecules can rotate rapidly about their long axes. These possibilities are illus--trated in Fig.49. There is no evidence, yet, for the transfer of lipid molecules from one side of the bilayer to the other ('flip-flop').

The physical state of the phospholipids depends very much on their temperature. At certain temperatures a change of state occurs from the crystalline (or gel) state to the liquid crystalline state, which is assoc--iated with increased conformational freedom for the lipid fatty acid chains. This state change is often referred to as 'melting'. The temperature at which

this melting takes place is a function both of the length of the fatty acid chains (the temperature is directly proportional to length, and to the degree of saturation), and also of the chemical nature of the lipid polar head groups[163] . Below the transition (melting) temperature, the fatty acid chains are packed in highly ordered hexagonal arrays[103] . Motion of the fatty acid chains in this gel phase is highly anisotropic and restricted[355] . In the liquid cryst--alline phase, the lipids still adopt a bilayer structure, but the arrays are very much more disordered, this disorder being attributable to bond rotation about C-C bonds. Typically, C-C bond oscillation frequencies are $5x10^{12}.sec^{-1}$[219] but there is a 'jump' frequency (rate of change from trans to gauche con--formation) of approximately $10^{10}.sec^{-1}$. So, there are approximately 500 vibrations within a given con--formation for each jump. The steric hindrance to rotation about a C-C bond will vary throughout the length of the fatty acid chain[344] , for example, rotation at a bond near the polar head-group will cause the rest of the lipid chain to rotate along the locus of a cone, and will thus be hindered by steric interactions. There are two possible mech--anisms for reducing these interactions and thus allowing increased disorder and increased motion towards the beginning of the chain. Firstly, motion in neighbouring chains might be correlated, so that

intramolecular rotation in neighbouring chains would occur to a similar extent; or, motions about several C-C bonds within a single chain might be correlated, so that the regular packing of neighbouring chains would not be greatly disrupted.

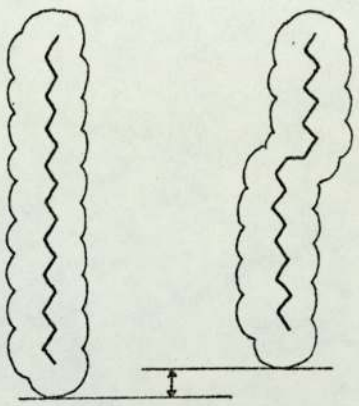


Fig.50:
Effect of a 2g-1 kink.
[from Lee[219]]

Rotation about two C-C bonds in a single chain (to the gauche conformation) gives rise to a 'kink' in the chain (the 2g1 kink[411] ), and this will decrease the length of the chain, as shown in Fig.50 (above). The number of kinks present in the liquid crystalline phase can thus be estimated from changes in the bilayer thickness and volume at the transition[411]. The formation of such kinks will be a dynamic phenomenon, with kinks forming and disappearing at any one position in the chain. However there should be more freedom for them to occur nearer the terminal methyl ends of the chains. Such motion could be involved in the formation of 'statistical pores' which have been invoked to explain some transmembrane diffusion phenomena[154].

The motion in space of any one carbon is the resultant of the motion about all the C-C bonds between it and the glycerol backbone, so that motion will increase along the chain because of the increasing number of bonds about which rotation is possible.

Kink formation will tend to decrease the probability of axial rotation of the lipid, fatty acid chains as a whole (fig.509) which in dimyristoyllecithin layers, has been found to be in the order of $7 \times 10^9$ $sec.^{-1}$[219]. Spin-label studies of the same layers suggested a very marked increase in microviscosity from the centre of the bilayer to the glycerol backbone region[362].

Because of the greater dissorder towards the ends of the aliphatic chains, the volume occupied by a $-CH_2-$ group towards the centre of the bilayer will be greater than that for a $-CH_2-$ group near the glycerol backbone region. The increase in volume might be accommodated in two ways: firstly there could be a bend in the fatty acid chains, with the upper portion tilted with respect to the bilayer plane; or secondly, there could be decreased packing density in the glycerol backbone region, perhaps with the extra space being taken up by water. There is some experimental evidence to support the former suggestion[258]. On average the bend occurs at about bond 8 and persists for approximately $10^{-8}$ sec., but it would be misleading to think of any long lived, or stable, net bend in the chains, just as it would be misleading to think of long-lived 2g-1 kinks. Whether or not the lipid fatty acid chains can be described by a bent chain model it is clear that the packing in the glycerol background region of the lipid bilayer must be 'looser' in the liquid crystalline phase than in the gel phase.

It seems likely that the extra space involved would be taken up by water molecules, although there is no real evidence to support this idea.

The transition between gel and liquid crystalline phases in lipids is generally very sharp, and this sharpness shows that the change of phase is a cooperative phenomenon. In this respect it is unlike a chemical transition, in which the equilibrium composition varies in a smooth and continuous way with change in a variable such as temperature. The sharpness of a phase transition depends on the number of molecules forced to co-operate in the transition (because of molecular interactions) and this group of molecules is called the 'co-operative unit' of transition[219]. A sharp transition would only occur for an infinite co-operative unit, and real crystals contain many flaws, each of which can serve as a nucleus for local melting, so that the co-operative unit will be much smaller than the total solid. Thus, at transition temperatures there will be small quasi-crystalline clusters of molecules showing co-ordinated movements present in the liquid phase, together with freely dispersed molecules[114]. Such clusters, called cybotactic groups, have been detected in various liquid crystals[97], and also in biological membranes[111, 219].

As well as the fast internal motions within lipid molecules in the liquid crystalline phase, there is strong evidence for a rapid diffusion of

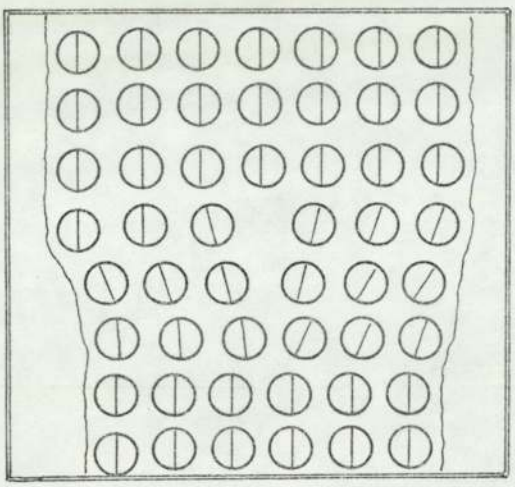


Fig.52:

A dislocation in a square lattice.

lipid molecules in the plane of the bilayer[219], the most likely mechanism for this being the movement of lipid molecules to temporarily vacant sites in the lattice (fig.51)[69]. Such vacancies might be considered as defects in the lattice, but there are other types of defect which are probably more important in diffusion. For example, a dislocation might exist such as in Fig.52. (opposite) where a line of molecules suddenly terminates. Around the dislocation line the crystal is strongly disturbed. Rows of such dislocations often occur in crystals and are called mosaic or sub-grain boundaries. A grain boundary is a line between two crystalline grains at which there is a large change of orientation, whereas a mosaic boundary is one where there is only a small change. In these regions, the energy needed to form a vacancy, or to move a molecule into a vacancy, will be lower than for the rest of the lattice, so that these regions can act as 'pipe' along which diffusion is greatly enhanced[283]. Whether these faults are present in mammalian cell membranes is not clear, but the presence of some sort of defect is highly likely on topological grounds. For a membrane to form a continuous surface enclosing a space, such as a

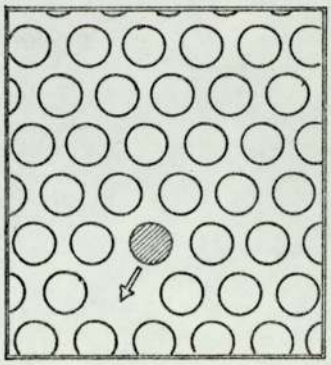


Fig.51:
Diffusion by exchange of position with a vacant lattice site.

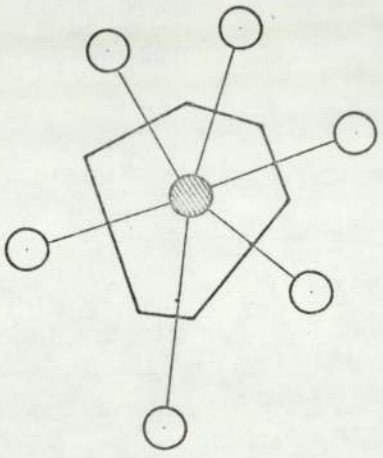


Fig.53:
The construction of a Voroni polygon.

typical cell, it must have a two dimensional curvature and this is not, in general, possible for a perfect two dimensional lattice. The easiest ways of accomodating curvature are by the introduction of lattice dislocations or by bends at grain boundaries.

Whilst the gel phase needs flaws for flexibility, the liquid crystalline phase is much less ordered, and faults are not so definite. A description of such a liquid is made in terms of the geometric neighbours of each molecule (Fig.53), and the construction made by bisecting the lines joining a molecule with each of its neighbours is called a Voroni polygon. The surface of a two-dimensional liquid can be considered to be made up of the Voroni polygons associated with each molecule in that surface. Topological considerations show that the average number of neighbours of any given molecule is six, and so the average Voroni polygon will have six sides[103]. (There must be one seven sided polygon for each five sided one). However, the fundamental characteristics of a liquid crystalline state, like those of a fluid, are the irregular arrangements of molecules and a continual change in their neighbourhood relations[31]. This interchange gives rise to the high fluidity of liquids and to self diffusion in the plane of the liquid[383].

Naturally, the composition of the lipid phase can have a profound effect on its behaviour. It is generally agreed that the addition of chloresterol to lipids in the gel phase 'fluidizes' the lipid

fatty acid chains whereas the addition of cholesterol to lipids in the liquid crystalline phase decreases the motional freedom of the chains[309]. If the cholesterol molecule is arranged with its 3β-OH group at the lipid water interface, then the first seven or so $-CH_2-$ groups of the lipid fatty acid chains will be in contact with the bulky ring regions of the cholesterol molecule, and thus will be restricted in motion. However, the cholesterol interacts preferentially with regions of high fluidity, so in biological membranes containing low concentrations of cholesterol, the cholesterol will not be randomly distributed when both gel and liquid crystalline lipid phases are present in the membrane, but it will be preferentially associated with those lipids which are in the liquid crystalline phase[219].

We thus imagine cell membranes to be composed of lipid and protein. The bulk of the lipid is assumed to be in the bilayer form, with a fraction of it possibly immobilised around the intrinsic proteins. Regions of the lipid will be in the gel phase and others will be more fluid. It is assumed that some proteins will span the membrane, whilst others will not. The lipids and the proteins are both capable of rapid motions about and within the plane of the membrane, resulting in a dynamic equilibrium structure for the membrane as a whole.

## XII Membrane Permeability

A living cell must not be isolated from the outside environment. It must be responsive to outside stimuli and it must be capable of absorbing nutrients and ions; but these proceeses have to be concerted and controlled in such a way that the cell remains aloof from its supporting medium even though it is completely dependent on it.

The simplest and paradoxically the most perplexing problem, is that of the membrane's selective permeability to small organic molecules and inorganic ions. Movement of molecules across cell membranes is movement from one aqueous environment to another and is restricted, therefore, to movement of solute molecules and of water. Gases such as carbon dioxide and oxygen, which are important in cellular metabolism, pass in and out of the cell in the dissolved state; and water itself exchanges rapidly across membranes. The existance of a concentration gradient of solute molecules across a membrane tends to cause a net movement of solute molecules in the direction of this gradient. The most useful model for this diffusion exchange appears to be that in which a small molecule is assumed to dissolve in the bilayer and move across by diffusion[457]; but a temperature study of non-electrolyte permeation across red cell membranes has shown that there are at least two barriers to membrane permeation. One provided by the water-membrane

interface and one by the membrane interior[129]. An intuitively attractive model for the diffusion of a small molecule across the membrane would involve the small molecule first entering the hydrocarbon centre of the bilayer through a lattice vacancy or 'transient pore' and then diffusing through the more fluid part of the hydrocarbon region in a pocket of free volume[220].

In fact the permeability of pure lipid bilayers to ions is generally very low. These low permeabilities presumably reflecting the large electrostatic free energy needed to bring an ion from aqueous solution (dielectric constant ca.80) into a lipid phase of low dielectric constant ($\epsilon$=2-3), which can be estimated to be ca. 46 Kcal. $mole^{-1}$. Papahadjopoulos[306] has suggested an increased permeability through the regions of disorder at the boundaries between discrete solid and liquid domains co-existing within the plane of the membrane. If permeability is greater in these boundary regions than in either gel or fluid domains, then a maximum in diffusion would occur at the mid-point of the phase transition, where the fractional area of the boundary regions within the membrane is greatest.

Selectivity of permeability does not seem to be a property solely of biological membranes, nor a function of complexity. Vesicles of phosphatidylserine and phosphatidyl glycerol exhibit a significant discrimination between the alkali metal ions, the

maximum $K^+/Na^+$ diffusion rate ratio being 10 for phosphatidylserine vesicles at pH.7.4[304]. In fact addition of chloresterol to these vesicles reduces the discrimination. Similarly, the permeability of simple lipid vesicles to Ca is less than to the alkali metal ions, but the permeability depends markedly on the nature of the lipid fatty acid chains. In dipalmitoyllecithin there is a marked increase in permeability at 40-50°, and the permeability of dioleoyllecithin vesicles is considerably greater than those of dipalmitoyllecithin, particularly at higher temperatures.

However, biological membranes are very rarely simple in their lipid composition. For example, sarcoplasmic reticulum, even though it posesses only one predominant protein (a $Ca^{++}/Mg^{++}$ ATPase), contains many classes of lipid which do not appear to be vital for enzymic activity (since simple liposomes of dioleoyllecithin with the ATPase are able to accumulate $Ca^{++}$)[430]. It is true, though, that the range of lipid complexity is necessary to prevent passive leak from the cells (which is excessive in the simple liposomes), and the lipid complex may well be vital to the rapid release of $Ca^{++}$ which must take place, after depolarisation of the membrane, if muscle contraction is to occur.

The permeabilities of lipid bilayers to metal ions can be increased dramatically by the addition of antibiotics such as valinomycin and nonactin.

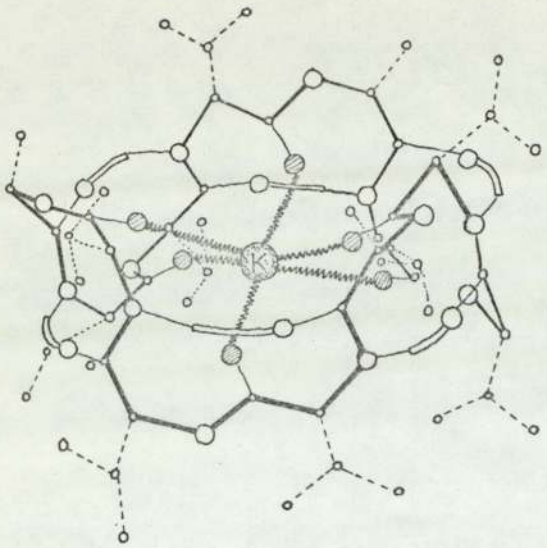


Fig.54 : Valinomycin-$K^+$ complex.

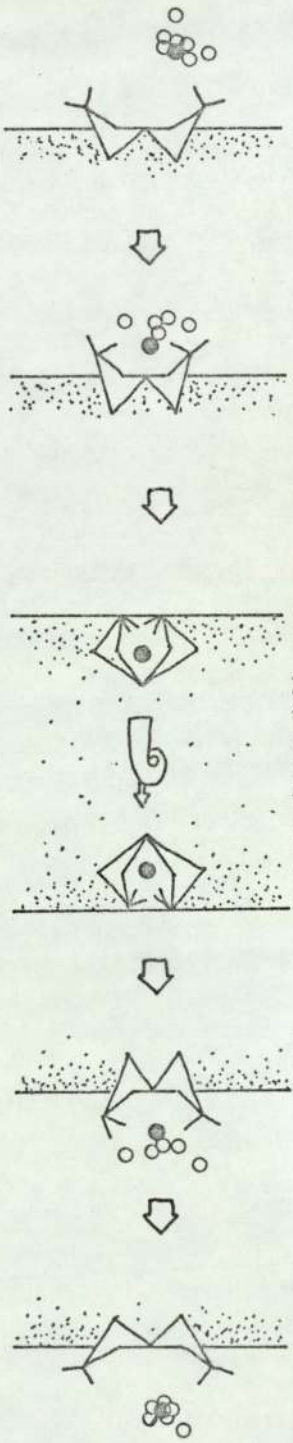



Fig.55: Mode of action.

The valinomycin gradually folds around the potassium at the lipid-water interface. During this process the water molecules associated with the ion are replaced one by one as it becomes coordinated with the oxygen atoms of the valinomycin [⊘].

(After Finean et al.[119] )

Valinomycin is particularly specific for potassium ions, and the structure of the $K^+$-valinomycin complex is such that the $K^+$ is contained within a polar environmemt within the complex, whilst the outer surface of the complex is hydrophobic[375] (Fig.54). It appears that the valinomycin gradually folds around the ion at the lipid-water interface. During this process the water molecules associated with the ion becomes coordinated with oxygen atoms in the ionophore (Fig.55) . This stepwise displacement of water of hydration from the ion reduces the potential energy barrier to penetration into the membrane.

Studies of the valinomycin-mediated ion movement across lipid membranes under the influence of a range of electrochemical potentials have indicated that the complex diffuses as an independent entity through the lipid barrier. Furthermore, movement through the hydrocarbon phase of the membrane, although influenced to an appreciable extent by the nature of the lipid[90], is more rapid than movement through the phase boundaries. Thus in this purely lipid system the rate-limiting step appears to be at the water-lipid interface as the ions are moved individually as ion-carrier complexes.

In contrast to this carrier mechanism for ion transport, the characteristics of ion movement through lipid bilayers induced by the non-cyclic polypeptide gramicidin A are more consistent with single file movement through channels. It has

therefore been suggested that several gramicidin molecules combine to form a water-filled channel through the lipid barrier[119]. These 'pores' appear to be less sensitive to the state of the lipid fatty acid chains than are the mobile carriers[156].

The specificities of membrane permeability to small molecules and ionic species are vital to the survival of the cell. However, the membrane is also sensitive to ions, particularly metal ions, in a number of other ways. The arrangement of the phospholipid bilayers of a biological membrane, with polar head groups oriented at the lipid-water interface, means that an electrostatic field will extend out into the surrounding water. This field will be relatively small for Zwitterionic phospholipids, but will be appreciable for a lipid carrying a net charge[219]. If the bilayer contains negatively charged phospholipids, for example, then the effect of the electrostatic field will be to attract positively charged ions into the vicinity of the interface between the lipid bilayer and the aqueous solution. This will produce an electrical double layer, in which the charge of the membrane surface is screened by a clathrate of counter ions of opposite charge. However, counter ion adsorption onto the charged surface is also possible, resulting in a charge neutralisation. In general, these two effects might be expected to go together, with some ions binding to the surface and thus partially neutralising the

surface charge, the remaining surface charge giving rise to an electrical double layer[86]. Calcium appears to be very much involved in the formation and collapse of these double layers. It has been shown that there is a low association constant for $Ca^{++}$ with Zwitterionic phospholipids, but a high one ($10^4$) for negitively charged phospholipids such as phosphatidylserine[370]. Measurements of these (apparent) association constants can only be approximate, but it appears that at low charge densities the order of apparent association constant with the cell membrane is $Mg^{++} > Ca^{++} > Sr^{++} > Ba^{++}$, whereas at high charge densities the order changes to $Mg^{++} > Ba^{++} > Sr^{++} > Ca^{++}$[22]. These interactions of metal ion with lipid bilayers have been shown to have important effects on the packing of the lipid molecules of membranes. They strongly suggest that divalent ions are absorbed onto specific sites on the membrane, whereas the alkali metals probably are not. Hence the concept of calcium binding sites in the membrane (and 'Group 1' calcium stores)[127].

This binding metal will affect the state of ionisation of the membrane, and an increase in the ionisation of the membrane lipids will cause a decrease in the melting temperature (because the electrostatic free energy of the bilayer in the liquid crystalline phase is smaller than in the gel phase), so that an increase in surface charge will favour the liquid crystalline phase[410]. Thus, the addition of $Ca^{++}$ or $Mg^{++}$ to dimyristylphosphatidic

acid causes a marked increase in the transition temperature. The effects occur at too low concentrations to be explained by screening within the electrical double layer and indicate that specific adsorption to the surface is probably important. The effects of divalent ions increase with increasing pH., suggesting that the divalent cations interact preferentially with doubly ionised phosphatidic acid, although some interaction occurs with the singly ionised forms[410]. (Phosphatidic acid has two ionisable protons, the first ionizing between pH. 1 & 3 and the second ionising between pH. 7.5 and 10).

Particularly interesting effects can be seen when $Ca^{++}$ is added to just one side of a membrane (for example, to a bilayer of phosphatidyl serine[295]). Adsorption of $Ca^{++}$ to one side of the bilayer will tend to neutralise the charge on that side, thus creating a difference in the surface energies between the two sides. Under these conditions groups of molecules will invert from one side of the membrane to the other. In so doing, the permeability of the membrane will increase, and, under extreme conditions, the bilayer will break[295]. As might be expected if this effect is caused by $Ca^{++}$ binding, increasing general ionic strength reduces the effect of $Ca^{++}$[271]. Similarly, addition of local anaesthetics, such as procaine, which displace $Ca^{++}$ from membranes, also inhibit the effect of $Ca^{++}$[294].

Of course, the permeability characteristics of cell membranes are not purely a function of the state of their lipids. The proteins associated with the membrane play a major role which is vital to the integrity of the cell, but even here there is no doubt at all that the activities of many of the (intrinsic) proteins are very closely controlled by the state of the lipid surrounding them. The ATPase activity of the calcium transport protein from sarcoplasmic reticulum has been found to be very sensitive to the state of the lipid surrounding it, and it is clear that the protein exerts no ATPase activity when its surrounding lipids are in the gel phase[430,431]. These phase transitions are usually detected using esr or fluorescent probes, but the work is complicated by the fact that the probe molecule will probably be largely confined to the more fluid part of the membrane. However, this difficulty can be overcome by modifying membrane fluidity by growth supplementation; then observing its effects. For example, it has been found that the activity of B-galactoside transport system of E.coli is very susceptible to phase transitions when grown in a medium enriched with elaidic acid[230].

There is quite strong evidence for a shell of 'immobilised' lipid around the membrane-bound proteins, and this immobilised lipid is assumed to have different properties from that of the bulk of the lipid matrix. Many proteins, if they are extracted from

the membrane, are found to have associated lipids, and if these lipids are removed, the biological activity of the proteins is often lost[289]. Further, it is highly probable that some segregated pools of lipid will be in the gel phase, whereas others will be in the liquid crystalline phase because, generally, the temperature range of gel to liquid crystalline phase transition encompasses the growth temperature, so that lipids in both gel and liquid crystalline phase are likely to be present[111,444].

There are clear indications that there is an important effect of lipid cluster formation on enzyme activity, for example, on the $Ca^{++}/Mg^{++}$ ATPase from sarcoplasmic reticulum. Here there is a sharp discontinuity in the rate of release of accumulated $Ca^{++}$ as induced by the antibiotic X537A, at 25° which correlates with a change in the state of part of the lipid complement of the membrane. Even in mammalian cells, where most of the lipids would be expected to be in the liquid crystalline phase at body temperature, the possibility exists that the cholesterol present in the membrane will associate preferentially with particular classes of lipid, which could cause a separation into domains of lipid clusters of differing fluidity.

There is an assymetry, too, between the two sides of the membrane, not only in the protein distribution, but also in the pattern of lipids (in fact the protein distribution is very likely to be

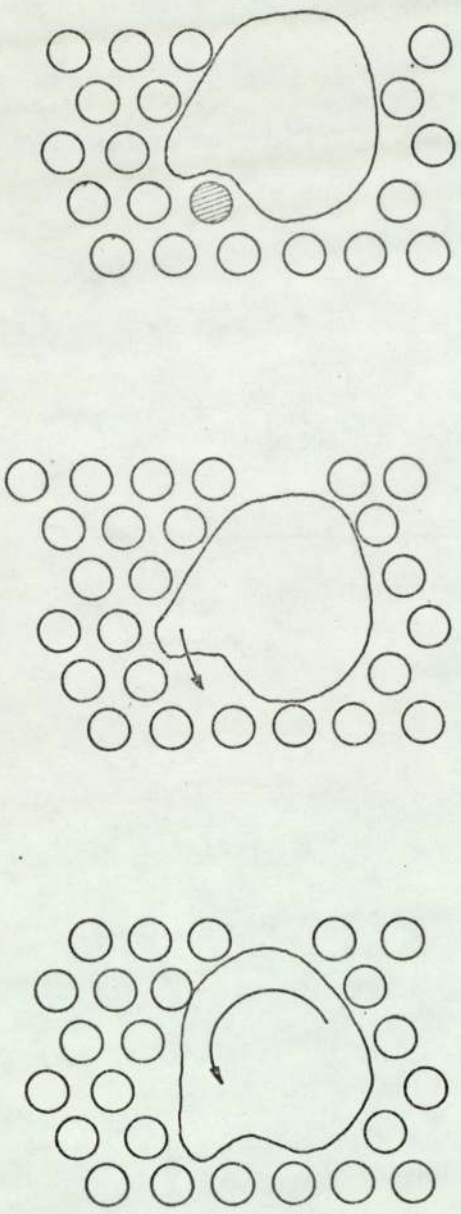


Fig.56:
Rotation of an intrisic protein in the plane of the membrane caused by the formation of vacancies in the lipid lattice.

a function of the lipid distribution). Bretscher[52] has shown this to be true for red blood cells, and the concept has been expanded to explain several aspects of the biological responses of cell membranes, in the 'bilayer couple' theory of Sheetz and Singer. It is very unlikely that there is much interchange (flip-flop) of lipids from one side of the bilayer to the other, (see Fig.49) but within the plane of a bilayer there is very rapid movement of most of the lipid molecules, (of the order of $10^{-8}$ $cm^2$ $sec.^{-1}$ in bacterial membranes[352]). This means that a lipid molecule can move from one end of a (1μ.long) bacterium to the other in a matter of minutes.

This lipid mobility gives rise to a motion of intrinsic proteins within the plane of the membrane, in a manner analogous to Brownian movement, but the only accurate measurement of a protein diffusion rate in the surface of a membrane that has so far been reported is that for rhodopsin in the retinal rod membrane[319] where a diffusion coefficient of $4 \times 10^{-9} cm^2 sec^{-1}$ was determined. The same work showed that the protein was freely rotating, about its own axis, in the plane of the membrane, at the same time; and this rotation is probably caused by the appearance and disappearance of vacancies in the lipid matrix of the membrane in positions next to local protrusions in the protein (Fig.56)[412].

Naturally, with such a high rate of mobility,

there is a strong possibility of proteins colliding with each other in their passage round the cell surface. The collision frequency between rhodopsin molecules is in the range $10^5$-$10^6$ collisions per sec.[319], but the significance of these collisions is not clear. It has been suggested that many of the electron-transporting systems, such as the electron transport chain, may not exist as a complex, or a fixed array, but be randomly distributed and free to move. Thus, translational movement of the cytochromes within the membrane would be required prior to their interactions. With mobility of proteins to this extent there is a problem in explaining the marked polarity of many cell types (for example, nerve cells, hepatic cells and skeletal muscle fibres). This polarity might be brought about by the aggregation of protein classes, which then cause a sympathetic, corresponding localisation of particular categories of lipid; or might well be established by the anchoring of certain proteins in the membrane by the internal microtubules, for example, in polymorphonuclear leucocytes where this has been shown to be true for Concanavalin A receptors, the distribution of which is disturbed by colchcine, which is known to disrupt microtubules[30]. However it is achieved, it is clear that this long range ordering of the membrane components is vital to the survival of the cell. This, together with the assymetric distribution of the lipid and protein between the

two sides of the bilayer, and the lack of homogenicity in the lipid phase distributions, is likely to be the key to the understanding of the activity of many membrane processes which are vital to the well-being of the organism.

## XIII Trans-membrane potential

As has already been stated, water exchanges rapidly across cell membranes. When solutions seperated by a membrane differ in concentration the diffusion of water in one direction exceeds that in the other. This results in an osmotic pressure which must be balanced (in the case of a cell) by some opposing pressure. This opposing pressure is the trans-membrane potential.

Cells include proteins and other immobile macro-molecules which at physiological pH. have a net negative charge. If charged macromolecules are present then an equivalent number of oppositely charged mobile ions must remain with them in the compartment in which they occur. This then leads, at equilibrium, to an unequal distribution of mobile ions across the membrane, which is described as a Donnan equilibrium[138]. When the activities of the mobile ions on either side of the membrane are unequal they set up an electrical transmembrane potential, the Donnan potential, and it is the balance between this potential and the osmotic pressure which establishes the ionic configuration

of a cell. Although the transmembrane potential can have a direct effect only on charged solutes, it nevertheless modifies the passive movement of uncharged solutes because of tis harmony with the osmotic pressure.

In the erythrocyte, as in all cells, the existing transmembrane potential (E) is the result of the sum of an infinite number of pressures, but it can be reduced to a simple Donnan potential, and this has been done by Goldman[138], to produce a constant field equation

$$E = \frac{RT}{F} \ln \left[ \frac{P_C[C]_o + P_A[A]_c}{P_C[C]_c \quad P_A[A]_o} \right]$$

where R = gas constant; T = absolute temperature; F is Faraday's constant and $P_C$ and $P_A$ the permeability constants for cations and anions respectively. $[C]_o$ and $[A]_o$ are the concentrations (activities) of cations and anions in the medium, and $[C]_c$ and $[A]_c$ are those of the cell water. For the red blood cell, potassium and chloride ions are the only ionic species which display any appreciable movement across the membrane, and it has been found convenient, and justified to reduce the Goldman equation to;

$$E = \frac{RT}{F} \ln \frac{a[K]_o + [Cl]_c}{a[K]_c + [Cl]_o}$$

$$a = \frac{P_K}{P_{Cl}}$$

Hunter[169] has shown that in the normal cell $P_{Cl}$ is about 100 times greater than $P_K$, so, provided that

chloride is distributed passively, the membrane potential should be equivalent to the chloride equilibrium potential.

The work described in this section has been carried out with the collaboration of my friend and colleague Jim Morgan (of the Department of Biological Sciences, University of Aston). In particular, all the results relating to mitotic activity and the blockade of mitogens by agents which are discussed here are derived from his work. This collaboration has enabled wider per--perspectives to be used in the discussion of the results obtained with the use of the fluor--escent probe system than would have been poss--ible if this data, alone, was available.

## XIV Investigations into Trans-Membrane Potential

Since it was likely that changes in trans-membrane potential might be involved, in some way, in the cellular response to oestrogens, it was necessary to develop a method of observing these changes. The work was carried out with blood cells suspended in aqueous medium, and, since it would have been difficult to use the standard technique of micro-electrode implantation with this system, this approach was precluded. There is expertise within this department in the use of these electrodes, but the manipulative problems of lancing a lymphocyte, let alone a platelet, are very much greater than those encountered with, for example, squid axon. However, in 1974 the new class of carbocyanine dyes were investigated for use as potential probes. Waggoner and Sims[382] screened a range of these pigments (which had hitherto been used as fabric dyes) and found several which could be used to monitor changes in trans-membrane potential.

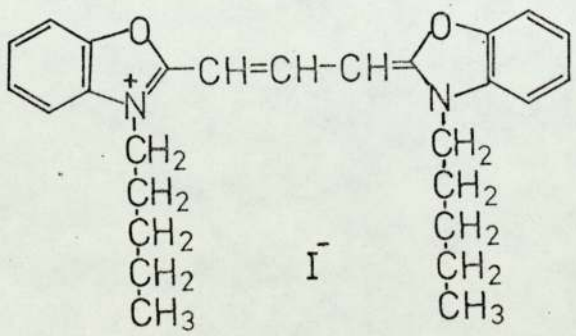



Fig.57

3,3'-Dipentyloxacarbocyanine iodide

[$CC_5$]

One of these dyes, 3,3'-dipentyloxacarbocyanide iodide (also called DiO-C5-(3) or CC5) (Fig.57) had excitation and emission characteristics which suited the instrumental parameters of the Aminco-Bowman fluorimeter used in this work, and this was selected for use in the subsequent experiments. Initially, a small supply of this dye was the very generous gift of Dr. Alan Waggoner; and subsequent supplies were synthesised according to a published method[382].

The carbocyanine dyes were investigated using a system of erythrocytes suspended in media of varying ionic strength, but attention had earlier been brought to bear on the cyanines when a merocyanine dye (Fig.58) had been incubated with squid axon membranes and been found to display changes in fluorescence which corresponded to changes in trans-membrane potential[87].

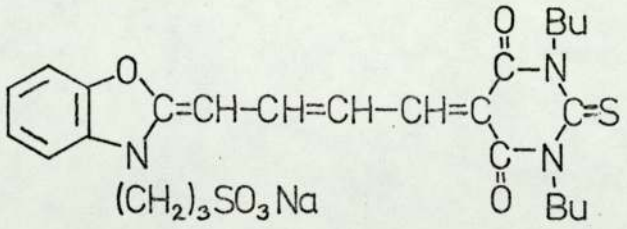



Fig.58: Merocyanine dye

Subsequently, Hoffman and Laris[165] determined membrane potentials in human and amphibian erythrocytes using a carbocyanine dye (Fig.59) which they compared with results obtained with standard voltage clamp techniques.

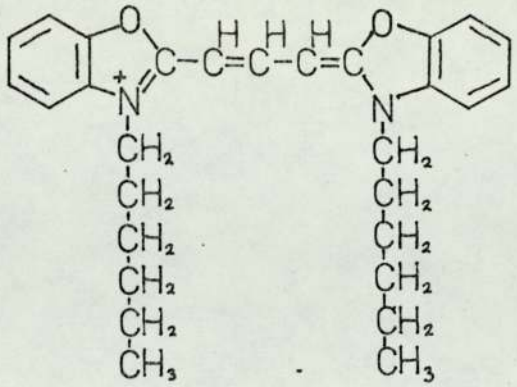


Fig.59: 3,3'-dihexyl-2,2'-oxacarbocyanine

Sims and Waggoner reviewed the carbocyanine class of dyes[382] and reported a wide range of investigations into the mechanism of their action. These authors came to no firm conclusions about the way in which the cyanine dyes sense trans-membrane potential, but they gave evidence to support the notion that the changes in fluorescence intensities, which are observed when the cell membrane potential is altered, result from changes in the degree of aggregation of the dye within the lipid palisade of the membrane.

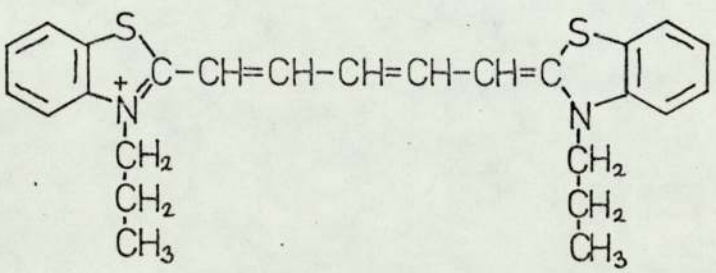


Fig.60: 3,3-dipropylthiodicarbocyanine iodide ($CC_6$)

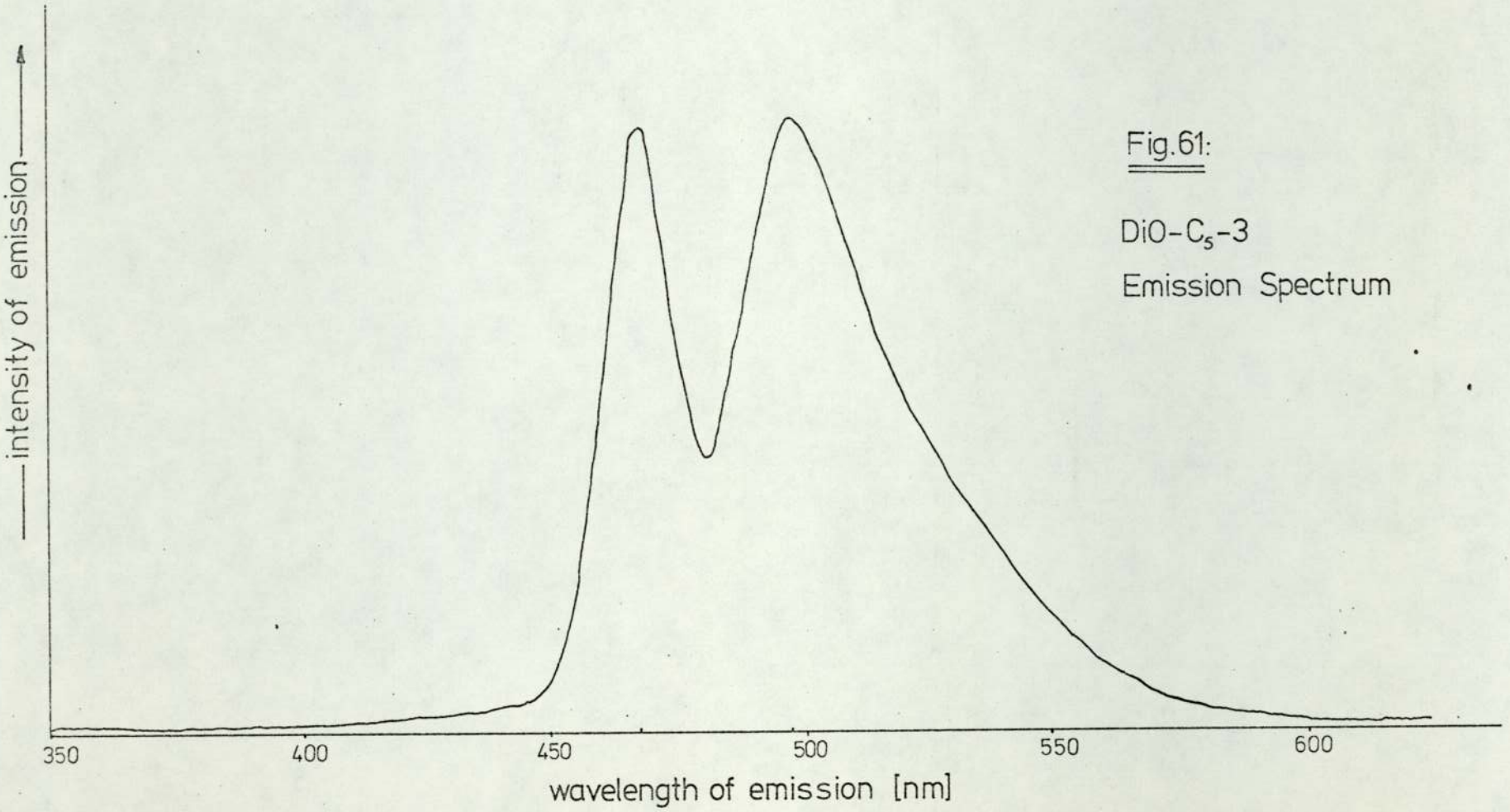



Fig.61:

DiO-$C_5$-3

Emission Spectrum

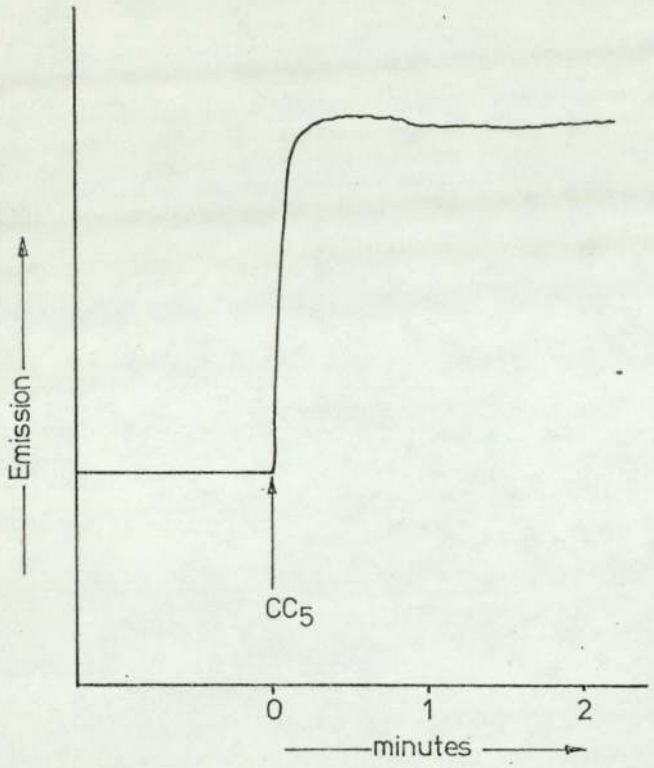



Fig. 62:

Addition of thymocytes to CC5 solution;

effect on fluorescence emission intensity.

With both CC5 and CC6 (Fig.60) the possibility exists for the positive charge of the fluorochrome to be distribute over the entire chromophore structure, which has two symmetric charge-resonance forms. The fact that the major charge is delocalised should reduce the free energy entailed in moving the dye from the polar medium into the hydrocarbon interior of the membrane, thus increasing the probablity of this occuring. When CC5 is dissolved in water, it displays a characteristic fluorescence emission spectrum (Fig.61), with excitation and emission maxima at 470 nm and 510 nm respectively. If the solvent is ethanol, the spectrum is qualitatively the same, but the intensity of emission is increased (by about two-fold). Thus, the dye fluoresces more strongly in organic solvents such as ethanol or octanol than it does in aqueous medium. If, now, a suspension of cells is added to an aqueous solution of the dye, there is an immediate increase in the fluorescence intensity (Fig.62), which suggests that the dye is moving to the hydrophobic area of the cell membrane from the aqueous medium, and is, as a consequence, showing a more intense emission inten--sity. The fluorescence changes which are observed when the cell membrane potential is altered result primarily from a potential-dependent partition of the dye between the cells and the suspension medium. If the inside of the cell is made more negative with

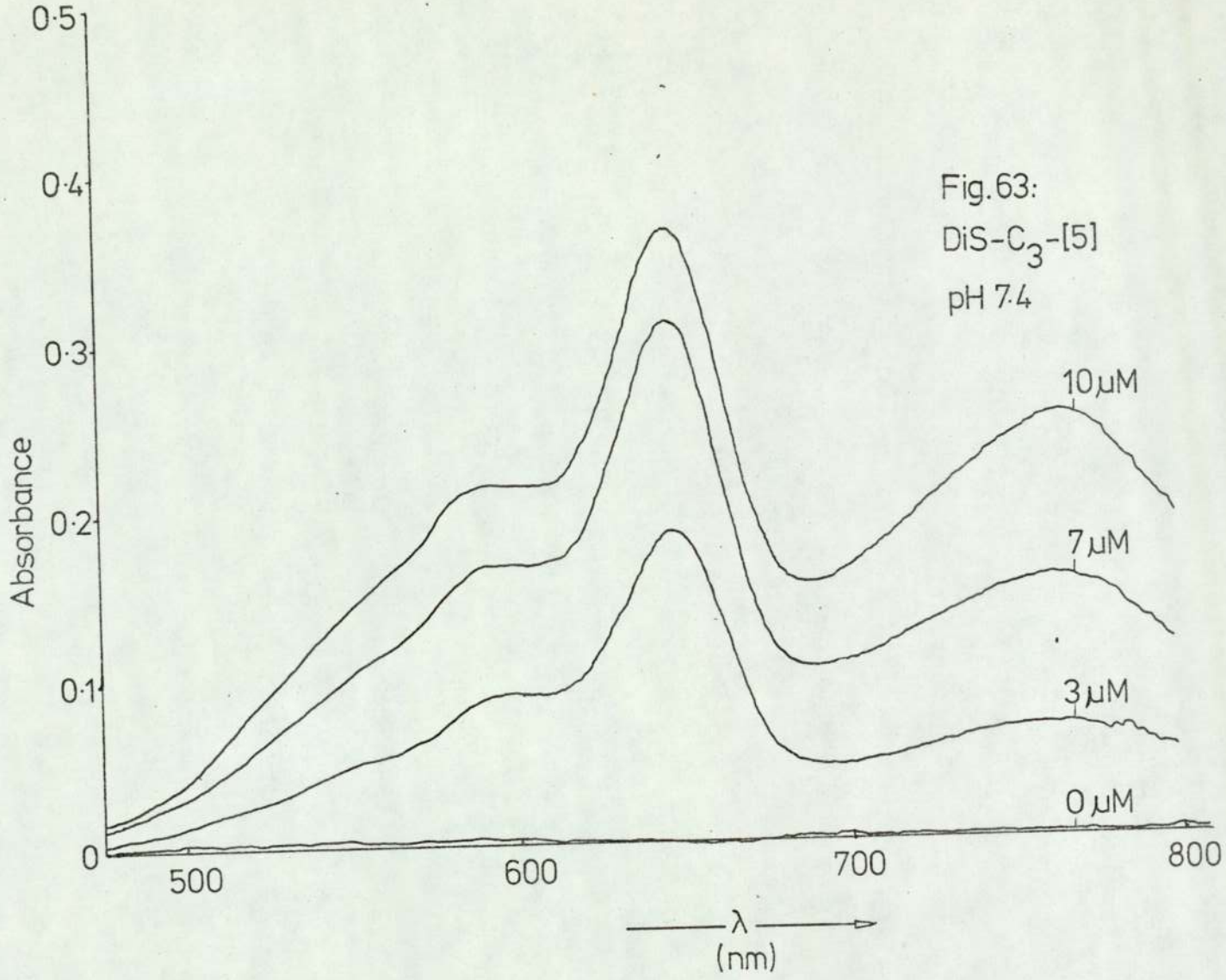



Fig.63:
DiS-$C_3$-[5]
pH 7.4

respect to the outside (hyperpolarised) the positively charged dye is likely to be attracted to the cell more strongly, and the equilibrium concentrations of the dye in the medium and in the cell shift more towards the cell as the membrane becomes hyperpol--arised.

Sims[382] suggested that, as the concentration of the dye in the plasma membrane increases, so the formation of non-fluorescent aggregates of dye within the membrane is favoured, and this has been offered as an explanation for the observed decrease in emission intensity which occurs on membrane hyperpolarisation. These workers concentrated on CC6 (Fig. 60), and support for this explanation is strong for this dye. The absorption spectra for CC6 (Fig.63) show that, under appropriate conditions, dimers and higher order polymers form as the concentration of dye increases. The principal (central) absorption peak is the absorbance of the monomeric dye, whilst the peaks at shorter and longer wavelengths are attributable to dimer and polymer forms respectively. The intensities of the side peaks increase, with respect to the central peak, as the concentration of dye increases, showing that polymerisation, with its resulting decrease in fluorescence, is favoured more as the dye concentration is increased, and this may well have a parallel with the increase in the concentration of the dye in the lipid phase of the

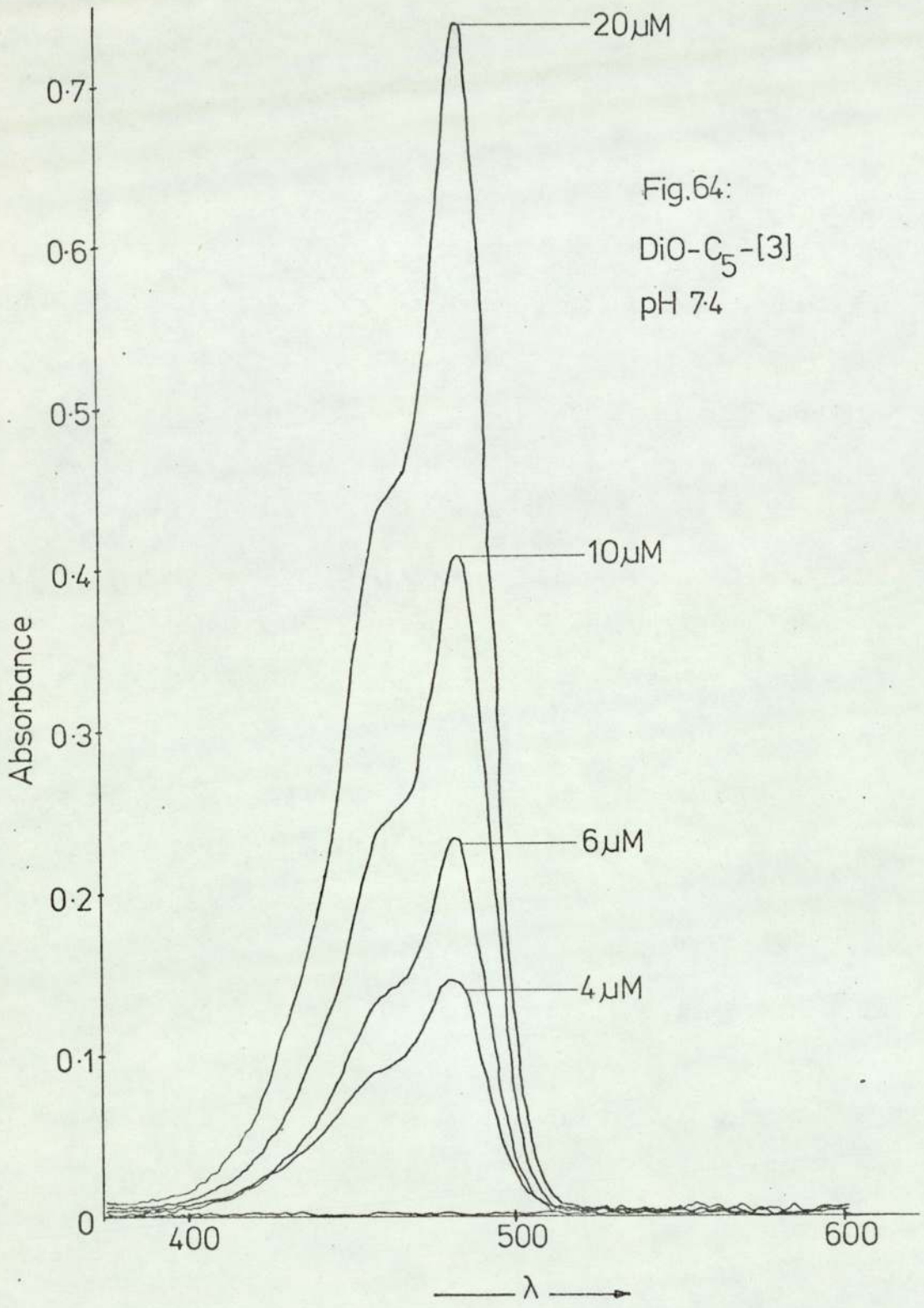



Fig.64:
DiO-$C_5$-[3]
pH 7·4

cell membrane which they suggest occurs with hyper--polarisation. However, Fig.64 shows that no similar aggregate formation can be demonstrated with CC5, despite experiments in a number of different solvent and buffer systems. For this, and for a number of other reasons, the system of dye distribution shown in Fig.65 has been adopted as a better working hyp--othesis for the interpretation of this work.

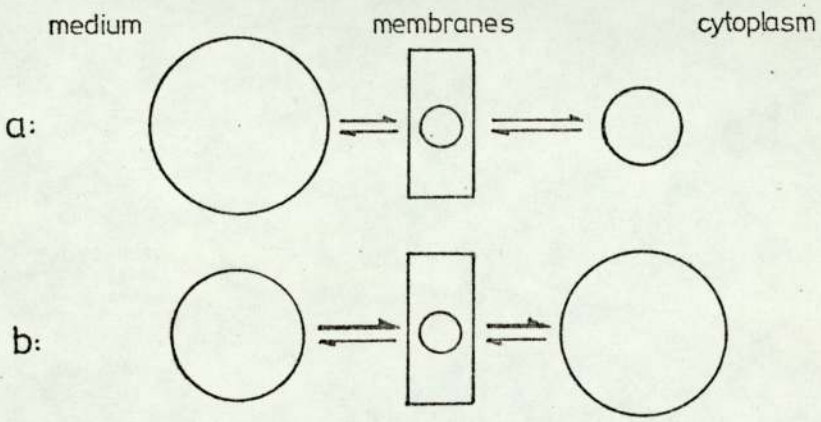



Fig.65:
Dye distribution changes on membrane hyper--polarisation. Equilibrium conditions (a) show circles denoting proportional dye con--centrations, and (b) the effects of hyper--polarisation.

In the hypopolarised cell, a large amount of dye is resident in the extracellular medium; some is dissolved in the membrane lipid, and only a small proportion of the dye is located in the cell cytoplasm. As the cell becomes progressively more hyperpolarised, the inside of the cell becomes more negative with respect

to the outside, and more dye is attracted into the cytoplasm. There is a corresponding decrease in the amount of the dye in the extracellular medium, and a resulting decrease in fluorescence intensity. The amount of dye resident in the lipid matrix probably remains relatively constant throughout the change in polarisation. The validity of this approach has been established in many experiments with cell-free systems as well as with lysed and aged cells.

At the time this work was undertaken the carbo--cyanine dyes were the only class of fluorescent probes which were thought to be of any use in monitoring membrane potential changes. 1,8-ANS (Fig.66) was tested since it had been used to study proton fluxes[19] but this dye does not recognise potentials in the same way as does CC5.

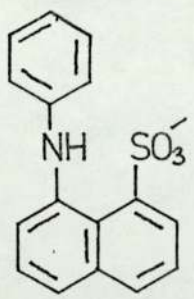



Fig.66:
8-anilino-1-napthalene
sulphonic acid (1,8-ANS)

ANS is thought to reside on the membrane in a region involved in ion transport, and has been used in specific studies on calcium movements across mito--chondrial membranes[70], in which changes in its

Fig.67:

Suspension medium for cells:

| | |
|---|---|
| Sodium chloride | 120.00 mM |
| Potassium chloride | 5.40 mM |
| $CaCl_2.2H_2O$ | 0.60 mM |
| $MgSO_4.7H_2O$ | 1.00 mM |
| $NaH_2PO_4.2H_2O$ | 1.03 mM |
| Glucose | 5.56 mM |
| sodium bicarbonate | 8.93 mM |
| HEPES | 20.00 mM |

(HEPES: (N-2-hydroxyethyl-piperazine--N'-2-ethanesulphonic acid)

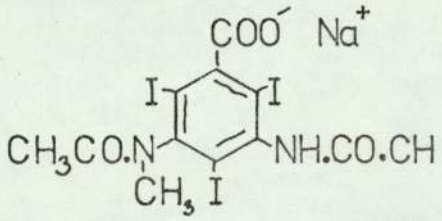



Fig.68: Sodium metrizoate

fluorescence characteristics were probably brought about by the interaction of the ANS with membrane ATPase[85] . The $CC_5$, had only been characterised for use with red blood cells, and erythrocytes are far from typical cells, being coated with a carbohydrate 'fur', and also being very highly specialised terminal cell forms with no nuclear potential. Accordingly, the first task was to establish that $CC_5$ could be used with cells other than erythrocytes, (subsequently papers have been published showing the results of carbocyanine dyes with mitochondria[217,403], and presynaptic nerve terminals[38] ).

All the cell types used were maintained in suspension culture in a modified Earle's salts solution (Fig.67), at a cell density of $5\times10^7$-$10^8$ cells per ml. Thymic lymphocytes were prepared in the following way: Using light ether anaesthesia, thymuses were removed from male albino rats (Wistar)weighing between 180-200 grammes before sacrifice. The glands were minced in medium, and the mince was subsequently filtered through moistened cheesecloth to remove any residual aggegates. The resulting cell suspension was diluted to the appropriate cell density with fresh medium, temperature being maintained at 37°.

To obtain splenic lymphocytes, spleens excised from male (Wistar) rats were extruded through a fine steel filter and minced in medium. Contaminating erythrocytes were removed from this cell suspension by isotonic shock in 0.76% ammonium chloride buffered

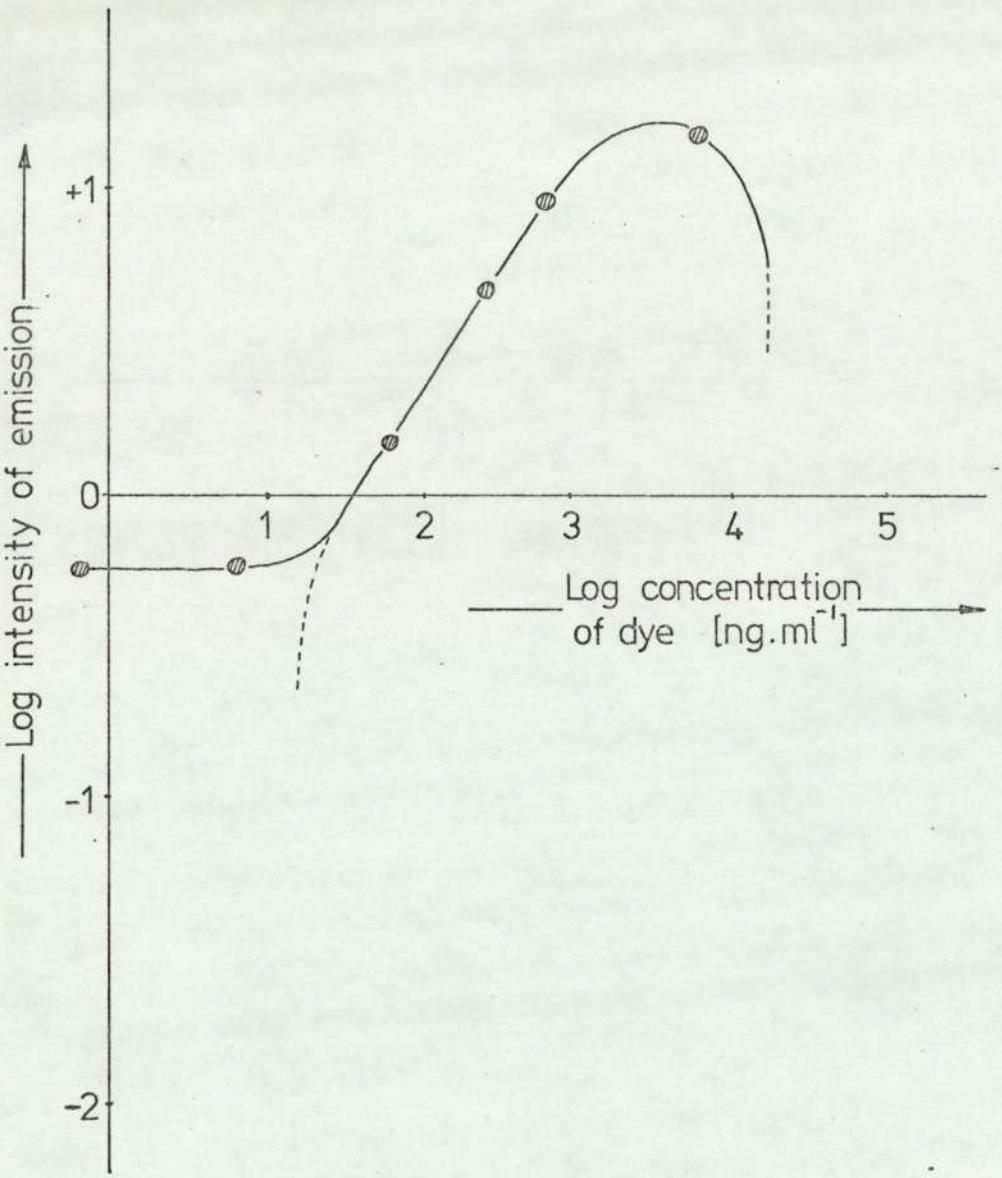
to pH.7.3 with Tris.HCl[46]. Following 2 washes the cells were suspended in medium at the appropriate density.

For the preparation of platelets, human venous blood was diluted 1:1 with phosphate buffer (ph 7.4) and aliquots of this solution were layered over a column which had been prepared previously by mixing 9% aqueous Ficoll 400 (24 parts) with 43% aqueous sodium metrizoate (3 parts), see Fig.68. The column was spun at 400 g. for 20 min., and the platelet layer was aspirated away and resuspended in medium. This suspension was centrifuged for 5 min., and the supernatant containing purified platelets, was diluted with salts solution and used in subsequent experiments.

When lymphocytes were used in mitotic assays they were maintained in Medium 199 (Burroughs Wellcome), containing no serum or antibiotics, and buffered to pH.7.2 with sodium bicarbonate. These cultures also contained the metaphase arresting agent Colchicine (Ciba Ltd), to a final concentration of 0.062 mM. Under these conditions the cells which flow into mitosis are arrested in a quasi-metaphase configuration where they accumulate linearly with time. After a 6 hour incubation, a sample was removed from the culture, fixed in neutral phosphate-buffered formalin, and stained with Delafield's haematoxylin[437]. The percentage of nucleated cells arrested in the so-called colchicine metaphase was then scored and

Fig. 69:

DiO-$C_5$-[3]



used as an index of proliferative activity.

All lymphocyte cultures were equilibrated for 30 min. prior to use. They were incubated in 1 ml. aliquots in plastic tubes (Sterilin Ltd) sealed with non-toxic stoppers, and were rotated at 40 rpm., about their long axes, in a roller drum assembly at 37° until required. For platelet experiments the platelets were used as soon as possible after preparation.

All fluoresence experiments were performed on a specially modified Aminco-Bowman spectrofluorimeter equipped, as before, with an EMI 9781B photomultiplier tube and 250W Xenon arc source (Englehard Hanovia Inc.). Emission outputs were fed directly to recorders or an oscilloscope display. Emission spectra were corrected using 'FLUCORR' where appropriate (see p.74 ). Typically, 3 ml. aliquots of cells, prepared as above, were stirred and maintained at 37° in a quartz 1 cm. light path cuvette within the instrument housing. The system was allowed to attain equilibrium after the addition of $CC_5$ dye to a final concentration of 2 uM, and the fluoresence emission intensity was recorded continuously during and after the addition of test reagents (normally in 30 ul. aliquots) to the cuvette. Using dye at a concentration of 2 uM ensured that small (local) changes in dye concentration would not affect the observed fluorescence quantum yield under cell-free conditions (Fig.69).

Ionophores and steroids were presented as

ethanolic solutions, after it had been established that addition of 30 ul. of ethanol to the cell system had no detectable effect on the fluorescence intensity. The fluorimeter monochromators were adjusted to give maximal response with median slit settings. Typically excitation and emission gratings were set to 470 nm and 510 nm respectively.

Absolute cell numbers (as assessed using a Coulter ZB1 cell counter) did not alter as a result of the incubation or fluorescence techniques. Similarly, cell viabilities (of lymphocytes) of 98%, determined using trypan blue dye exclusion, were maintained throughout the duration of the experiment.

In the earlier reports[165,382], the carbocyanine dyes had been standardised using suspensions of red blood cells exposed to valinomycin (see p.116 ). The addition of valinomycin produces a highly selective increase in K permeability in cell membranes[153], thus increasing the value of $P_K$ (page 125 ), the magnitude of which can even exceed the control value for $P_{Cl}$ . This increase in $P_K$ should result in a change in the trans-membrane potential from the chloride equilibrium to the potassium equilibrium potential. Thus, if potassium concentration is high within the cell (which is as true for lymphocytes and blood platelets as it is for erythrocytes), and potassium concentration is low in the supporting medium, then addition of valinomycin should produce a change in the trans-membrane potential, which

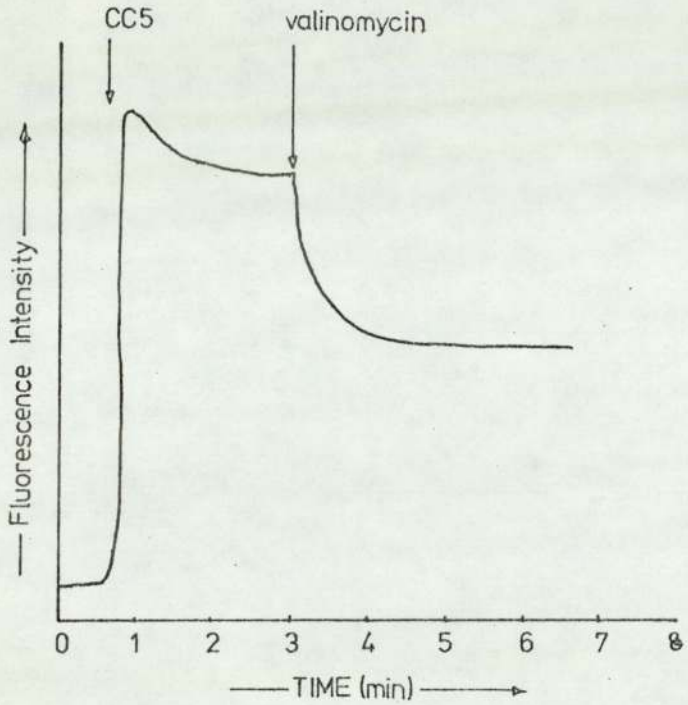



Fig.70:

Effect of adding valinomycin ($10^{-5}$M) to a stirred suspension of thymocytes, equilibrated with the dye CC5, on the fluorescence emission intensity of the system.

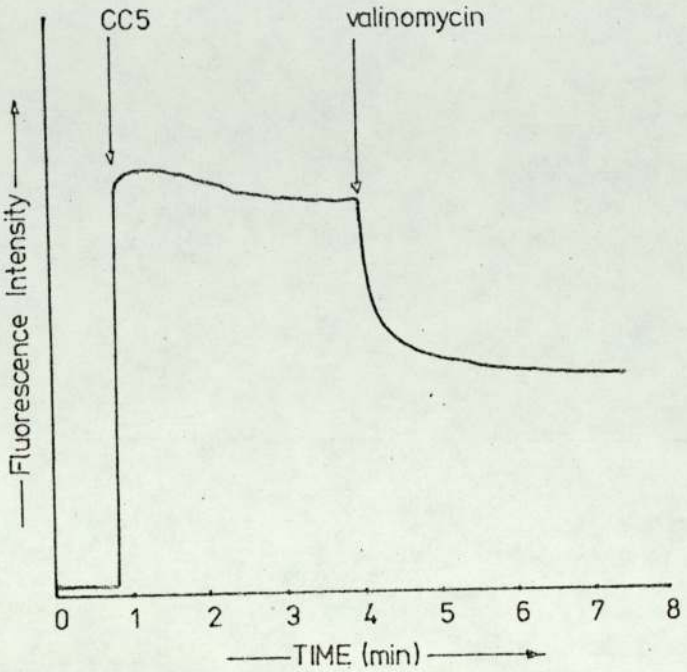



Fig.71:
Effect of adding valinomycin ($10^{-5}$) to a stirred suspension of splenic lymphocytes, equilibrated with the dye CC5, on the fluorescence emission from the system.

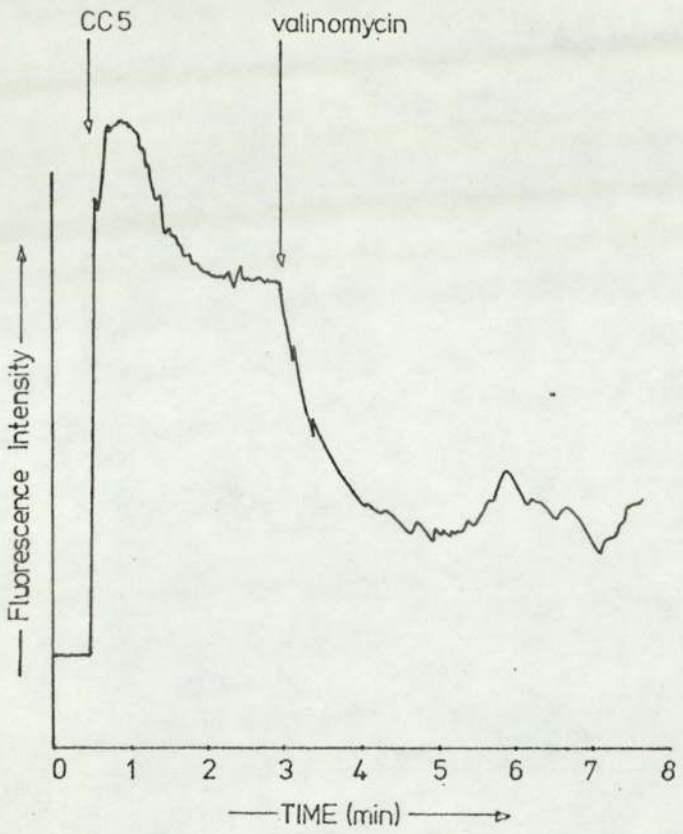



Fig.72:
Effect of adding valinomycin ($10^{-5}$M) to a stirred suspension of human platelets, equilibrated with the dye CC5, on the fluorescence emission from the system.

would be reflected in a fluorescence change. Fig.70 shows that valinomycin was added to thymocytes which had been equilibrated with carbocyanine dye there was a significant decrease in the fluorescence emission intensity. Essentially similar results were obtained when this procedure was repeated with splenic lymphocytes (Fig.71) and blood platelets (Fig.72). This suggested that $CC_5$ was suitable for use with these cell types. The establishment of a new (potassium equilibrium) potential across the membrane was causing a very rapid change in the transmembrane potential, which was being detected by the dye. That it was potassium which was the significant ion in this process was demonstrated when the changes in the fluorescence intensity were reduced when the extracellular potassium concentration was raised, and increased when it was lowered (Fig.73).

Since the transmembrane potential is a function of $\propto$ (see page 125), it is clear that a change in potential may be brought about by a change in membrane permeability to a particular ion, there being no requirement for that ion to be moved physically across the membrane boundary. Clearly, changes in potential resulting in membrane hyper- and hypopolarisation are likely to be a function not just of permeability changes, but also of forced ionic movements, and even of the 'streaming' potentials which are always associated with the motion of charged species such as ions . In order to establish the

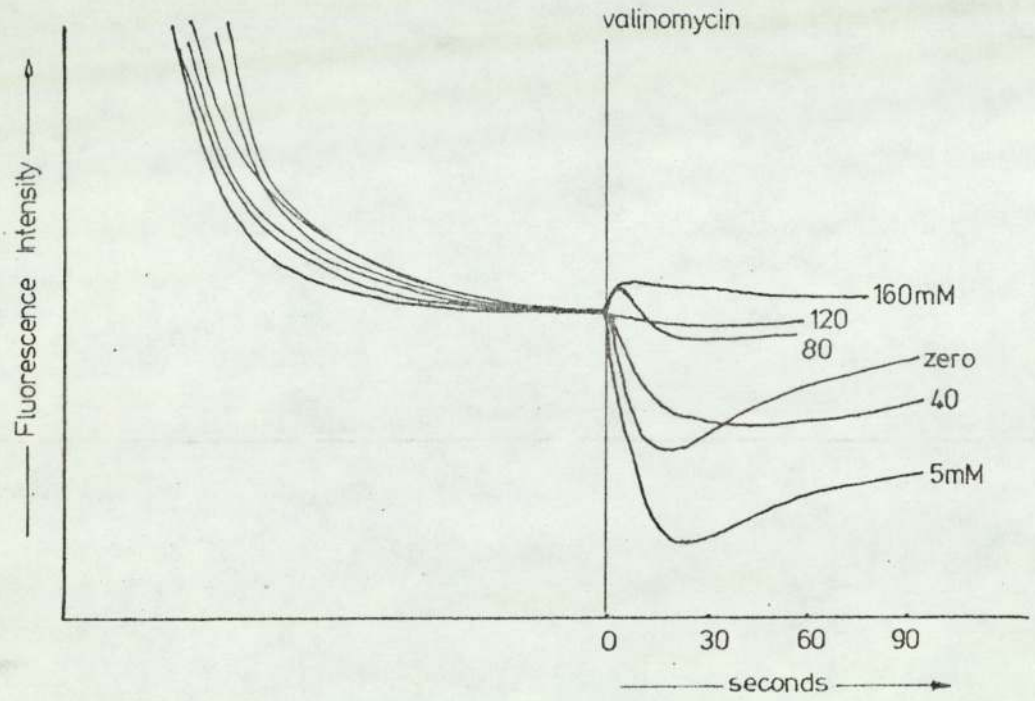



Fig.73:

Effect of adding valinomycin ($10^{-5}$M) to a stirred suspension of rat thymocytes, equilibrated with CC5, and with differing concentrations of potassium in the supporting medium, on the fluorescence emission intensity of the system.

existence of ion movement, as opposed to changes in permeability, precipitated by the addition of valinomycin, and other agents, the following technique was adopted: For uptake studies radioactive isotopes (typically $^{42}K^{+}$, $^{45}Ca^{++}$, $^{22}Na^{+}$, $^{32}PO_4^{---}$) were added to cell cultures to a final radioactivity of 1 $\mu Ci.ml^{-1}$ at time zero, together with the agent under investigation. Aliquots of this mixture (0.2 ml.) were immediately dispensed into polypropylene microfuge tubes containing 50 ul. formic acid overlaid with 100 ul. silicone oil (DC-550).

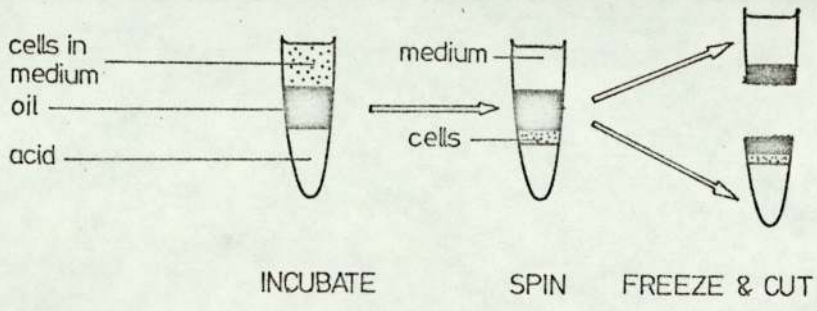



Fig. 74:

After the appropriate period at 37° the tubes were spun in a Beckman Microfuge at 12,000g. for 2 min., plunged into liquid nitrogen, and bisected so as to separate the lysed cells (in the acid phase) from the suspension fluid which lay above the inpenetratable oil layer. This method, amodification of that used by Freedman et al.[127], provided an excellent way of rapidly removing cells from their supporting medium. Radioactivity of tube fractions was determined on either a Tracerlab Gammaset 500 gamma emission spectrometer, or on a Beckman LS 230 liquid

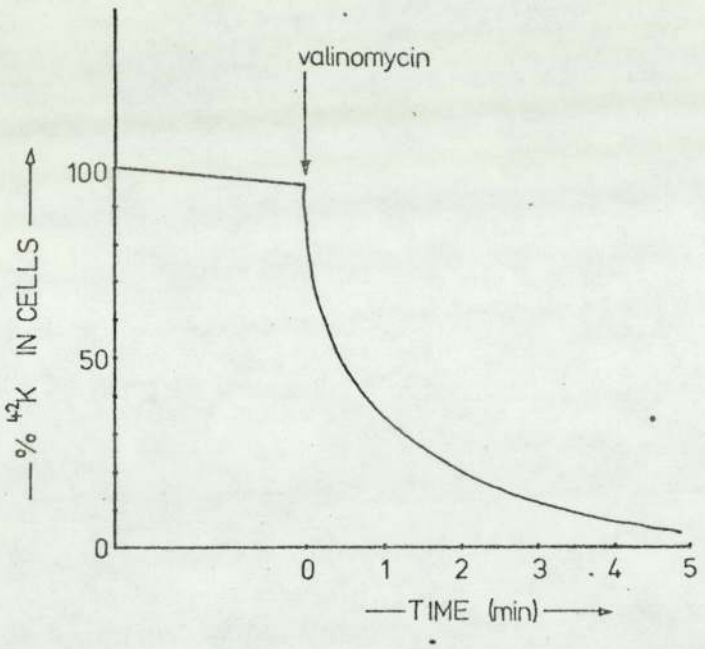



Fig.75:

Effect of adding valinomycin ($10^{-5}$M), to a suspension of thymocytes pre-loaded with $^{42}$KCl, on the rate of efflux of potassium.

scintillation spectrometer, using 5 ml.of NE 260 (Nuclear Enterprises) as scintillant fluor. For ion efflux studies, cells were incubated for 50 min. in the presence of 1 $\mu Ci.ml^{-1}$ of the different isotopes, after this period the cells were spun from suspension and washed three times in unlabelled medium to remove excess isotope. Subsequently cells were subjected to the agent under investigation as described above.

As Fig.75 shows, when valinomycin was added to thymic lymphocytes there was a marked efflux of $^{42}K^{+}$ from the cells which had previously both equilibrated with the isotope. These observations were repeated using $^{86}Rb^{+}$ as the tracer ion. Clearly, the membrane permeability to potassium is enhanced by valinomycin, so the prodominant effect will be the movement of potassium down its concentration gradient[16], which, if it is unaccompanied by anion efflux, or an opposing cation influx, will cause membrane hyperpolarisation.

Having established that valinomycin-induced hyperpolarisation could be detected with CC5 dye, the next step was to use other ionophores to promote membrane potential changes. Attention has already been drawn to the link between oestradiol activity and calcium[48], so the next logical move seemed to be to study the effects of calcium ionophores on the cell membrane potential. Two such ionophores were selected for study; A23187 and X537A.

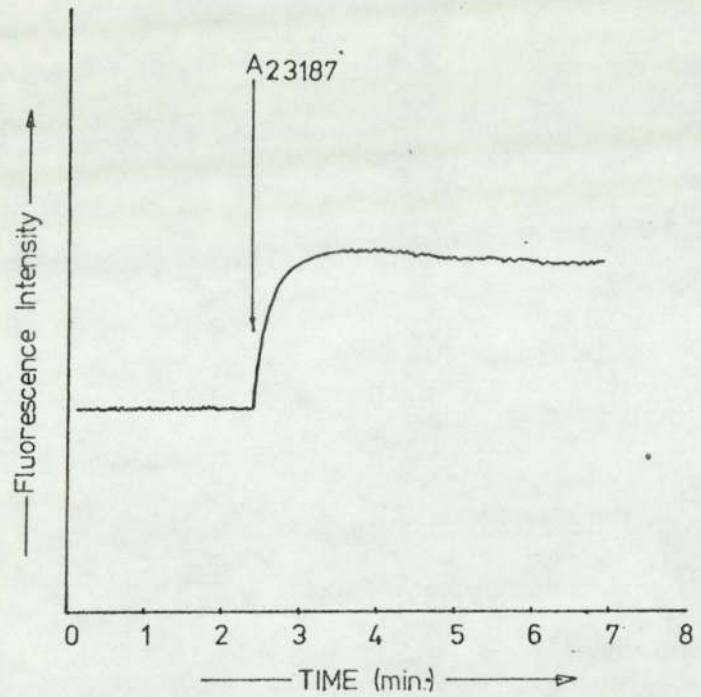



Fig.77:
Effect of adding A23187 ($10^{-5}$M) to rat thymocytes suspended in medium containing 0.6 mM calcium, and equilibrated with CC5, on the fluorescence emission intensity from the system.

The carboxylic acid antibiotic A23187 is a divalent cation ionophore which has been shown to uncouple oxidative phosphorylation and inhibit ATPase in rat liver mitochondria[331,445]. The compound is prepared from cultures of Streptomyces chartreusensis as a mixed salt, which can then be converted to the free acid. Its structure[71] is shown in Fig.76a, below:

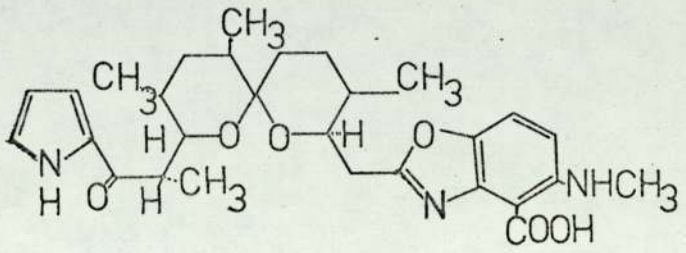


Fig.76a:
A 23187

X537A (Ro 20-0005/001) is another antibiotic obtained from an unidentified streptomycete[29] which promotes rapid release of accumulated calcium from sarcoplasmic reticulum vesicles[112,357] and which can thus exert contractile effects in muscle preparations[436]. Its structure is shown in Fig.76b, below:

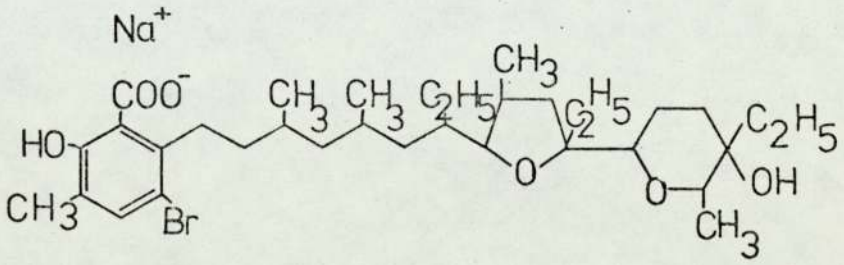


Fig.76b:
Bromo-X537A

The ionophores appear to act in much the same way as valinomycin (page 116). X537A, in particular, has been shown[186, 436] to form a dimer to surround the cation, which becomes stabilised by coordination and hydrogen bonding. The complex has a spherical shape, enclosing the ion in a hydrophilic pocket, while the exterior is non-polar and lipophilic, and, as a consequence increases the permeability of membranes to these divalent cations[320]. In line with this suggested mode of action (see Fig.55) is the observation that increased membrane fluidity by heating[9] or incorp--oration of benzyl alcohol[9,264] promotes the ion transport by the two ionophores.

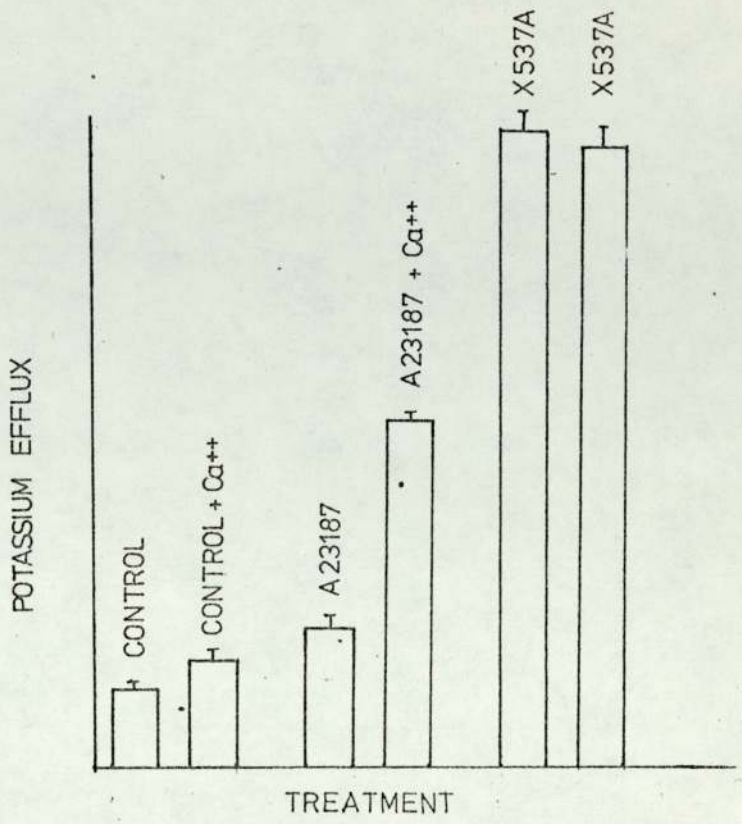
A23187 is the more specific ionophore, having higher affinity for calcium than magnesium, and rel--atively little affinity for other ions[332], whereas X537A binds and equilibrates both monovalent and divalent cations[321], and, as a consequence, in mixed ion media, the two compounds can promote rather different effects[339]. For this reason, A23187 was used in preference to X537A in much of the work described here.

When A23187 was used to increase permeability to calciumions, its addition caused a change in the fluorescence emission intensity (and hence a change in the trans-membrane potential) which was dependent in magnitude and direction, on the extracellular calcium concentration. This response is directly

| ION | | Con.A. | $A_{23187}$ | $Ca^{++}$ |
|---|---|---|---|---|
| $Na^{+}$ | IN | + | + | O |
| | OUT | N.T. | N.T. | N.T. |
| $K^{+}$ | IN | + | + | O |
| | OUT | + | + | O |
| $PO_4^{///}$ | IN | + | − | N.T. |
| | OUT | O | + | N.T. |
| $Ca^{++}$ | IN | + | + | + |
| | OUT | + | + | N.T. |

Table 6:

To show the effect of adding Con-A (5 $\mu g.ml^{-1}$), A23187 ($10^{-5}$M) or calcium (1.8 mM) to a stirred suspension of lymphocytes on the movements in and out of the cell of several ionic species.




Fig,78:

Effect of various treatments on the rate of potassium efflux from lymphocytes.

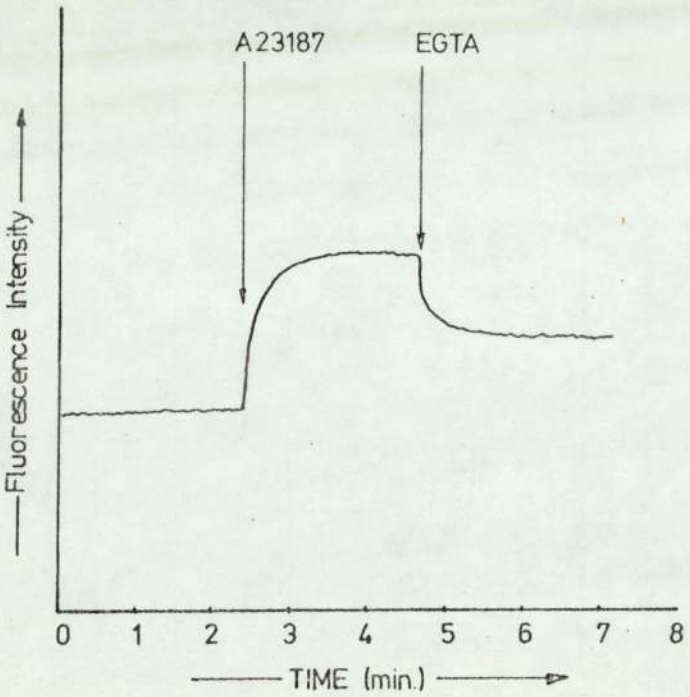



Fig.79:

Effect of the addition of A23187 ($10^{-5}$M) and the subsequent addition of EGTA on the fluorescence emission intensity from a stirred suspension of rat thymocytes, equilibrated with CC5, in a medium containing 0.6 mM calcium.

analogous to that observed with valinomycin, except that calcium is now the principal permeant ion. Fig.77 illustrates this result, and Table 6 shows that A23187 not only facilitated calcium movements but also dramatically stimulated the efflux of $K^+$ and $PO_4^{'''}$ as well as the uptake of $Na^+$ & $K^+$. Note that the membrane is hypopolarised when the A23187 is added, because the external concentration of calcium is very much higher than that of the cell cytoplasm. This is in direct contrast to the situation with valinomycin and potassium.

In the absence of extracellular calcium, A23187 promoted only trivial ion movements, (whereas the less specific ionophore X537A was not so calcium-dependent) as shown in Fig.78, and also promoted only very slight changes in fluorescence with the CC5/ lymphocyte system. Thus, the changes in trans-membrane potential precipitated by A23187, in the presence of calcium, is clearly a function of the concerted movements of several ions, and these movements are probably triggered by the action of calcium on the cell membranes. Fig.79 shows that these events can be modified by the subsequent addition of the specific calcium chelating agent ethylene-bis-(oxyethylene--nitrilo)-tetraacetic acid (EGTA). This has the effect of decreasing the extracellular concentration of ionised calcium (by chelation), and suggests that the trans-membrane potential changes precipitated by

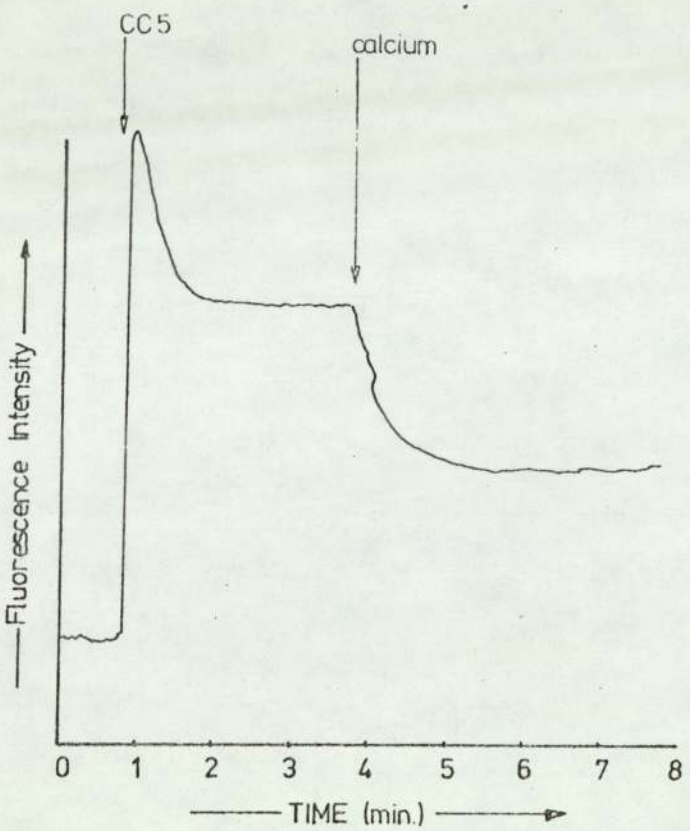



Fig.80:

Effect of the addition of calcium (1.8mM) on the fluorescence emission spectrum from a stirred suspension of rat thymocytes, equilibrated with CC5, in a medium containing 0.2mM calcium.

A23187 are calcium-dependent, and remain so for a period of some minutes. These responses are common to all the cell types studied but do not apply to aged or lysed cells. In sonicated preparations containing membrane fragments neither valinomycin nor A23187 induced any alteration in fluorescence intensity. Furthermore, in moribund cell cultures, in which normal transmembrane ion gradients have degenerated, no fluorescence changes could be elicited by the two ionophores.

Since calcium is clearly an important ion in the context of cellular response, the effect of calcium alone, with no ionophore present, was studied. After a 30 minute pre-incubation period in the absence of calcium, the addition of calcium to a final con--centration of 1.8 mM produced a marked decrease in fluorescence with a system of thymocytes and $CC_5$. The fluorescence reached a maximum value within 120 sec. (Fig.80). Thus, calcium addition established membrane hyperpolarisation which was assumed to be the result of a change in the membrane permeability character - istics. Smaller incremental additions of calcium re-vealed a progressive increase in hyperpolarisation, with the maximum rate of change of fluorescence in--tensity occuring between 0.2 and 1.1 mM calcium. Maximum intensity values were obtained at 1.8 mM calcium (Fig.81).

This hyperpolarising action of calcium could not have been an artefact of initial calcium deprivation during the pre-incubation period for when

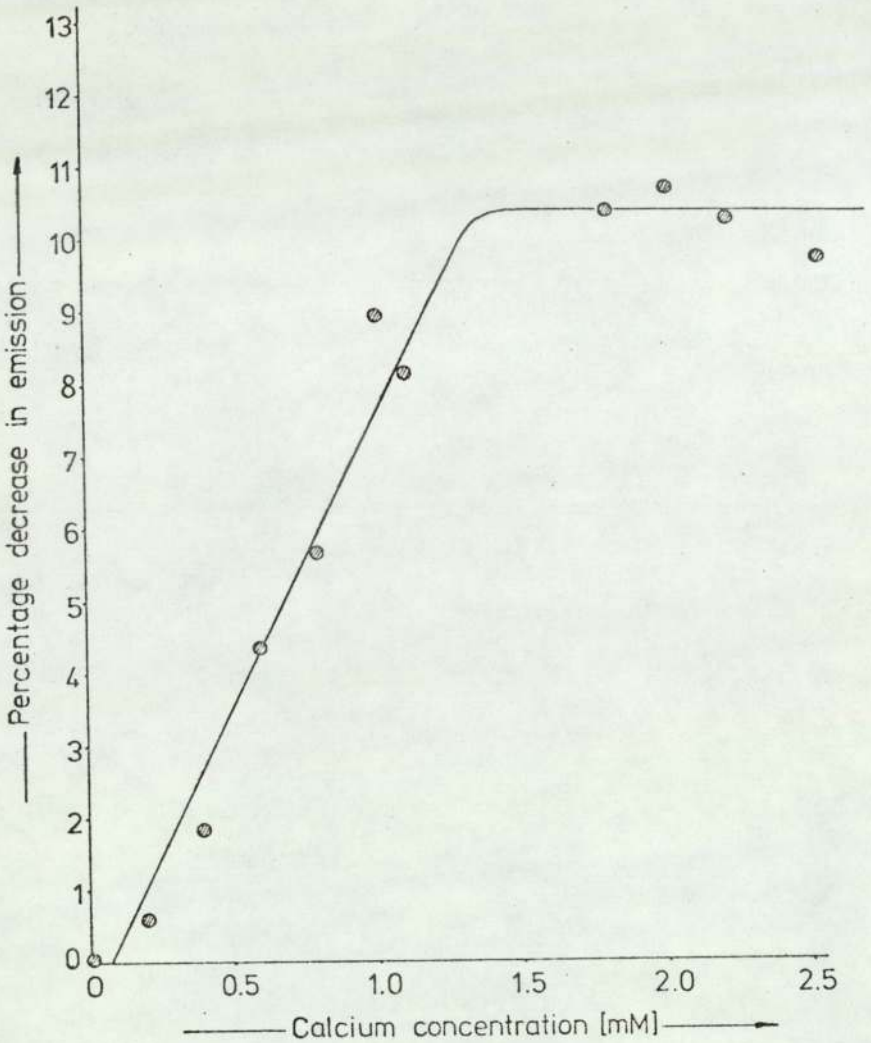



Fig. 81:

Progressive decrease in emission intensity (indicating a progressive hyperpolarisation) as the amount of calcium added to a stirred suspension of rat thymocytes, equilibrated with CO5, is increased. Calcium concentration of media 0.2 mM prior to calcium challenge.

Fig.82

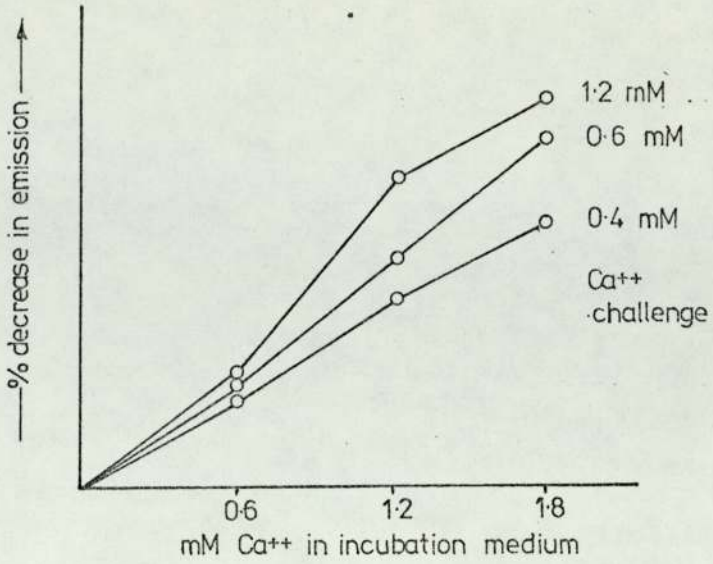
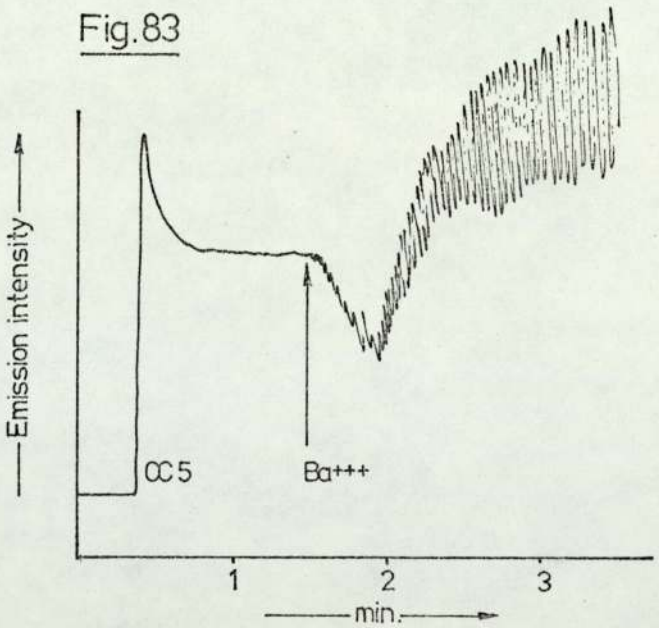

% decrease in emission
1·2 mM
0·6 mM
0·4 mM
Ca++ challenge
0·6
1·2
1·8
mM Ca++ in incubation medium

Fig.83


Emission intensity
CC5
Ba+++
1
2
3
min.

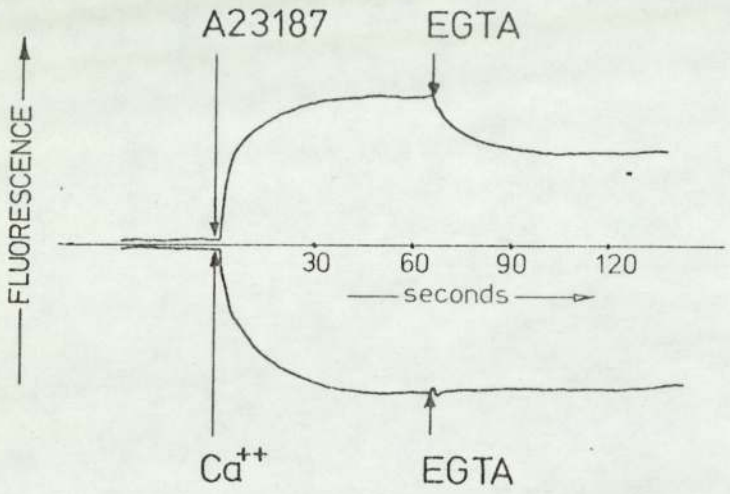



Fig.84:

Effect of EGTA on membrane hyperpolarisation triggered by A23187/calcium, and by calcium alone.

cells were pre-incubated with various concentrations of calcium, further calcium additions still induced hyperpolarisation (Fig.82). When the ion fluxes associated with this calcium challenge were studied, no clear pattern emerged however (Table 6). On a number of occasions calcium-triggered $^{42}K^{+}$ effluxes were noted, but the variability of the results was such that these effluxes could not be considered significant. Of the various cations tested only calcium induced a membrane hyperpolarisation. Magnesium, strontium, zinc, manganese and tin all had no effect on fluorescence when added to cells. Cadmium and barium caused fluorescence changes (Fig.83) but these were attributed to precipitate formation, and not to dye-related events. Magnesium, although not promoting a membrane potential change on its own, did appear to be capable of moderating (slighty reducing) a calcium induced fluorescence change if it was present at high (3 mM) concentrations. (Fig.91).

Ionised calcium values in plasma may vary between 1.0 and 1.2 mM under different physiological conditions; and the highly specific calcium-induced hyperpolarisation of the thymocyte plasma membrane which occurs over this concentration range (Fig.81 & Fig.82) may well represent a true physiological role for the ion in membrane function. Once established a calcium triggered potential change could not be reversed by the addition of EGTA (Fig.84). Thus calcium seems to initiate some event which subsequently

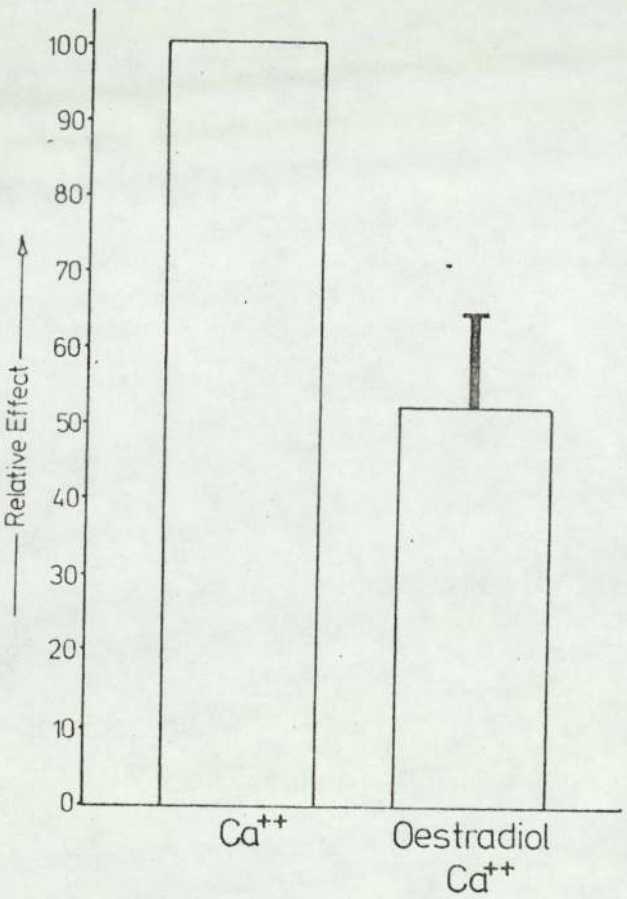



Fig.85:

Effect of calcium (1.2mM), and of calcium plus oestradiol ($10^{-6}$M) on the stimulation of mitosis in rat thymocytes.

(From Morgan et al.[275])

generates and maintains the observed potential alteration. Certainly it is not related to a simple diffusion of more calcium ions into the cell, for when such a movement was provoked, with high specificity, by the addition of A23187, it resulted in an increase in fluorescence (Figs.79 and 84) ie. hyperpolarisation. Furthermore, the hyperpolarisation which followed ionophore addition was at least partially reversed by EGTA addition.

Having developed a method, then, which was capable of detecting the transmembrane potential changes which occur on exposure of cells to calcium (a method which is much more sensitive than the ion flux technique), the activity of oestradiol was investigated in a similar system. Oestradiol had already been shown to inhibit calcium-induced mitogenesis in isolated thymic lymphocyte cultures[274,277] [Fig.85] and this steroid was added to the cell suspensions in the fluorescence system both before and after the addition of calcium ions. In each case, when oestradiol-17B (10 μM) was added to the cultures a small but significant hypopolarisation was observed (Fig.86). Furthermore, when calcium (1.8 mM) was added to the cultures after the oestradiol, the hyperpolarisation induced by the ion was reduced to some 50% of that in paired cultures containing no steroid (Fig.86). Thus oestradiol blocked both calcium induced mitogenesis[274] and hyperpolarisation. It was not established, however, whether the hyperpolarisation

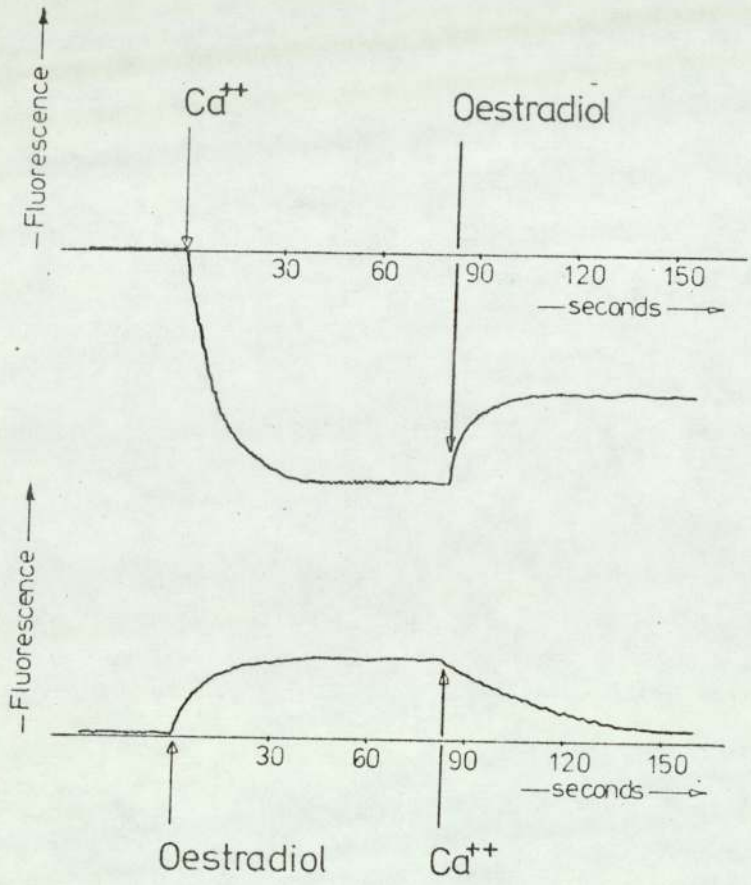



Fig.8b:

Effect, on the fluorescence emission intensity, of adding oestradiol ($10^{-5}$M), before and after the addition of calcium (1.8 mM), to suspensions of rat thymic lymphocytes previously equilibrated with CC5 dye.

(and associated ion fluxes?) which calcium causes were related to, or essential for, this ion's ability to recruit cells into their DNA synthetic phase. In an attempt to provide a partial answer to this question a range of steroids was examined to see whether an ability to inhibit calcium-induced mitogenesis was always associated with a capacity to block calcium induced hyperpolarisation.

Table 7 shows that several other oestrogenic molecules also had the ability to inhibit calcium-induced potential changes. A superficial analysis of these structures reveals that only 18 carbon steroids possessed this property, whilst 19 carbon androgens and 21 carbon progestogens were without activity. It is also a feature of the anti-mitotic steroids that they possess an aromatic A-ring, which might represent an important feature of structural activity. However, two synthetic derivatives of oestradiol, the 3-methyl ether of oestradiol and the 3-methyl ether of 17-ethyl oestradiol, lacked anti-proliferative activity. It is likely therefore that other aspects of structure are important in delineating this action of oestrogens. One such parameter may be the groups present on carbon atoms 3 and 17. Since the alpha epimer of oestradiol is equally as effective as the biologically active beta form of the steroid, this suggests that the spatial configuration of the carbon 17 hydroxyl is of no importance. Furthermore, it would indicate that the

TABLE. 7:

| | SUPPRESS MITOSIS | SUPPRESS $Ca^{++}$ TRIGGER | INCREASE FLUORESCENCE | DECREASE FLUORESCENCE |
|---|---|---|---|---|
| β-Oestradiol | + | + | + | / |
| α-Oestradiol | + | + | + | / |
| β-Oestradiol-3-benzoate | + | + | + | / |
| Oestriol | + | + | + | / |
| Oestrone | + | + | + | / |
| Oestrone-3-sulphate | + | + | + | / |
| 17α-Ethyl-β-oestradiol 3-methyl ether | / | / | + | / |
| β-Oestradiol 3-methyl ether | / | / | + | / |
| β-Oestradiol-3-glucuronide | / | / | / | / |
| β-Oestradiol-17-glucuronide | / | / | / | / |
| Testosterone | / | / | / | + |
| Androstene-dione | | / | / | / |
| Androstene-diol | / | / | / | / |
| Dehydroisoandrosterone | / | / | / | / |
| Progesterone | / | / | / | / |
| Cholesterol | / | / | / | / |
| Anisole | | / | / | / |

interaction being observed is not being mediated through classical cytosol receptors which show little affinity for alpha oestradiol[195,291] . It is also possible to replace the carbon 17-hydroxy function of oestradiol with a ketone group with little loss of activity. Also the additional hydroxylation of carbon 16 has no effect upon the ability of the steroid to inhibit calcium-induced mitogenesis. In terms of substitution at the carbon 3 position a sulphate group does not seem to impair function whereas a glucuronide does. This latter observation may relate to the poor lipid solubility of glucuronide forms of steroids[78] , an important feature in their excretion.

When carrying out a similar structural analysis for the ability to blockade calcium induced mitogenesis it was apparent again that only carbon 18 oestrogens were active. Thus a close resemblance was noted between requirements for activity in blocking calcium-induced mitogenesis and hyperpolarisation. Remarkably the biologically inactive alpha epimer of oestradiol was again equally potent, which also suggests a superficial site of action for the steroid. Furthermore, oestrone and oestriol possessed some activity as did the 3-sulphate derivative of oestrone. Indeed the only discrepancy between the two groups was the 3 methyl ether derivatives of oestradiol and the 3 methyl ether of 17-ethyl oestradiol which did block calcium triggered hyperpolarisation but not profileration. The 3 benzoate

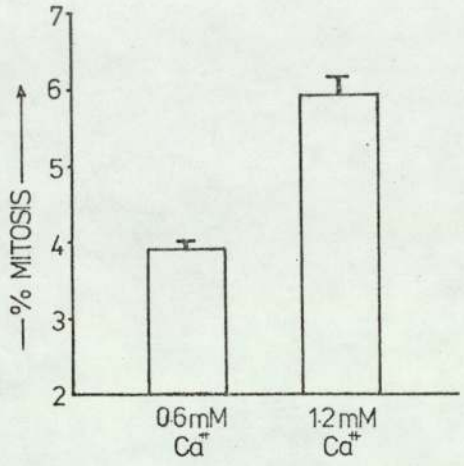



Fig.87:

derivative blocked calcium-induced mitogenesis whereas the 3-methyl ether did not. Since both com--pounds are particularly lipid-soluble it is unlikely that this property is a primary factor governing mitotic blockade. It is possible that the important features include the general property of lipid solubility, an aromatic A ring, and groups at positions 3 and 17 which are capable of some inter--action with elements of the membrane structure.

Parenthetically, it might be added that testo--sterone induced a small hyperpolarisation, an activity which might indicate some importance for this steroid in membrane function (Table 7) and which was in complete contrast to the small hypopolarisation observed with oestradiol.

As has been seen, when thymic lymphocytes are cultured in medium containing 0.6 mM calcium, a certain number of cells enter mitosis over a period of 6 hours. If, however, the concentration of calcium in the culture medium is increased to 1.2 mM, then an increased rate of cell division is noted[278] (Fig.87). Other experiments have shown that calcium causes the recruitment of a number of relatively quiescent cells into the DNA-synthetic stage (S-phase), which culmin--ates, a few hours later, in cell division. Oestradiol is able to suppress this activity[272] (Fig.85). Calcium, of course, is not alone in this respect. A wide variety of hormones and other agents can also stimulate mitosis in thymocytes, including:

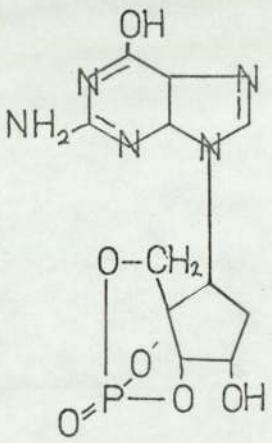



Fig. 88:

cGMP

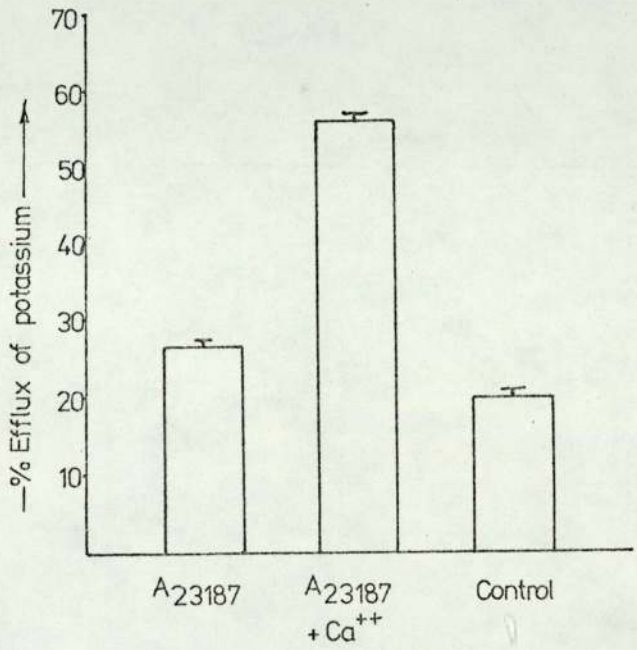



Fig. 89:

Effect of the ionophore A23187, in the presence and absence of calcium, on the promotion of potassium efflux from rat thymocytes.

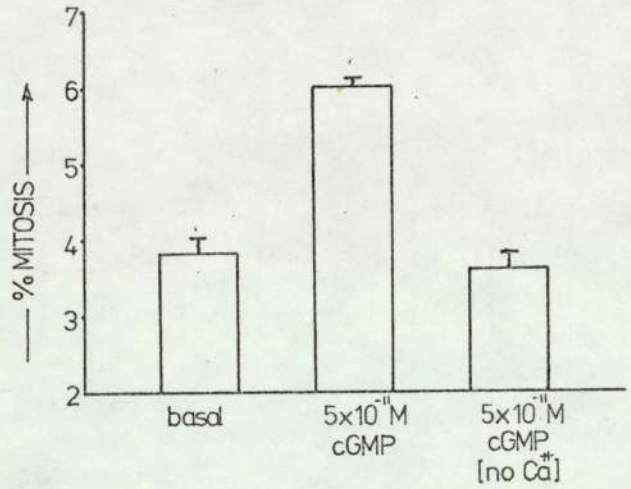



Fig.90a:

Effect of cGMP, both in the presence and absence of calcium, on the rate of cell division in rat thymocytes.

histamine (H-1), insulin, acetylcholine, Con-A, and A23187[49]. It had already been established that A23187, in the presence of calcium, promoted a mem--brane hypopolarisation; and the ability of A23187, and all the other agents, to stimulate $K^+$ efflux is entirely dependent on the presence of calcium. If no calcium is present they cannot exert their effects (Fig.89). Another feature common to these agents is that they are all known to elevate intracellular cyclic guanosine monophosphate (cGMP) levels when they are applied to cells (Fig.88)[136,170,214,364]. Further--more, cGMP itself, at a concentration of $5 \times 10^{-11}$M, will stimulate mitosis in these, and other, cells[369,439] and, again, this ability to increase the rate of cell division is wholely dependent on the presence of calcium (Fig.90a). (It is worth noting, as a rider, that cAMP also stimulates cell division in these cells, but that in this case the mechanism is magnesium dependent, and not related to calcium[49]).

The earlier results with calcium and A23187 had shown that the CC5 dye system was a good technique for the investigation of membrane responses to mitogenic agents, and since there was good reason for believing that the plasma membrane was playing a considerable rôle in concerting these responses, the effects of cyclic nucleotides themselves were studied with the fluorescent probe.

It was found that dibutyryl cGMP (dBcGMP), at

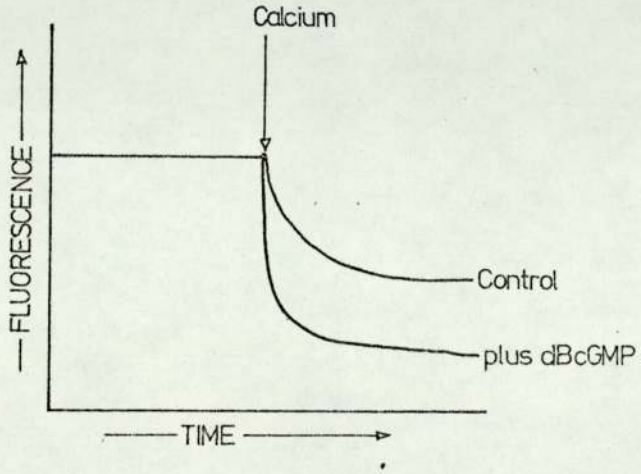



Fig.90b:
dBcGMP enhancing the response to calcium in thymocytes; as determined using the cell/ 005 fluorescence system.

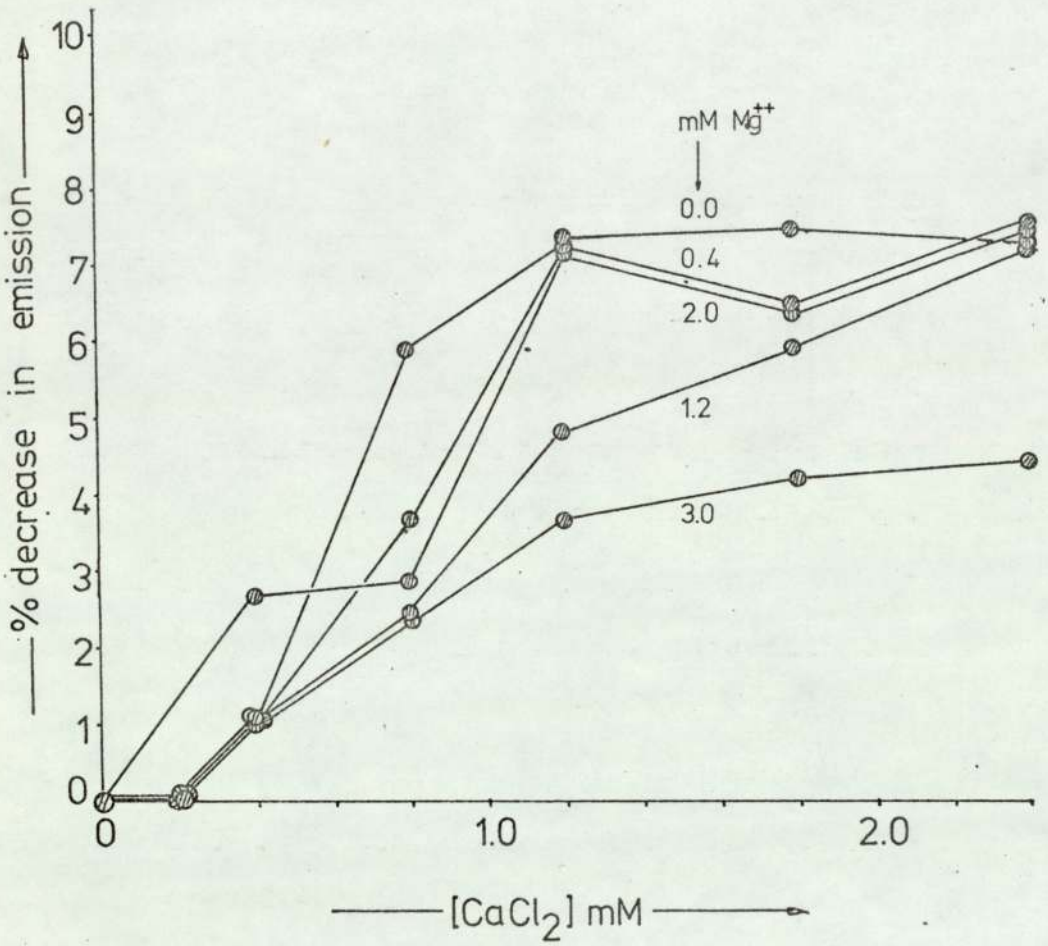



Fig.90c:

Effect of calcium added to cells incubated in various concentrations of magnesium; showing inhibition of hyperpolarising effect at high magnesium levels.

relatively low concentratios, enhanced the effect of calcium in promoting membrane hyperpolarisation (Fig.90b). The native cGMP also had this effect, although higher concentrations had to be used than with the dBcGMP, presumably because of the fact that dBcGMP enjoys easier access to the cell cytoplasm by virtue of its greater membrane penetrating properties.

It has already been noted (page 150) that cAMP stimulated cell division by a magnesium-dependent process. In the fluorescence system it was observed that, whereas cGMP enhances the calcium-induced hyperpolarisation, cAMP inhibited it, as did magnesium itself (Fig.90c). This may be yet another example of the much fêted reciprocity between the two nucleotides, and may well be important in the elaboration of the theory that it is not the absolute levels of the nucleotides which are of paramount importance, but the relative concentrations of each.

Thus, it appears that cGMP sensitises the cell membrane to external calcium, and facilitates the induction of membrane hyperpolarisation. It has already been demonstrated (page 146) that this effect cannot simply be attributed simply to an increase in the membrane permeability to calcium, so we are forced to the conclusion that ions other than calcium are promoted to move across the cell membrane, and that this movement can be triggered by the action on that membrane.

Table 6 shows that Con-A and A23187 both precipitate the movement of several ions across the plasma membrane. Movement of phosphate shows no clear pattern, and it is unlikely that phosphate plays a crucial rôle in the stimulus event. However, both Con-A and A23187 stimulate the movement of calcium across the membrane, and under physiological conditions it is clear that the net effect will be an influx of calcium into the cell. Thus, both the mitogens promote calcium influx, and since they both require calcium for activity, it seems probable that the activity of the agents is attributable to their ability to promote an influx of calcium into the cell.

In addition to calcium movements, Table 6 shows that, when the cell is challenged with the mitogens, sodium uptake is increased slightly, although the effect is small, and in fact it is very difficult to get enough labelled sodium into the cell to allow for a meaningful efflux experiment. Pre-treatment with cyanide and iodoacetate can be used in order to load cells with sodium$^{38}$, but it is unclear what effects this might have on the systems under inves--tigation. Potassium, however, is dramatically affected, both in efflux rate and uptake. It thus appears that sodium and potassium are the best candidates, with calcium, for casting as those ions responsible for the hyperpolarisation (and mitotic stimulation) following stimulus of the cell with

calcium, or a calcium-dependent agent. Use of the ATPase inhibitor ouabain (Fig.91) suggested that the first effect of calcium on the cell membrane is to increase the permeability to potassium, thereby causing membrane hyperpolarisation.

This phase is then probably counteracted by the action of a sodium potassium ATPase in the membrane, thus causing further ionic redistributions. All these changes, or their subsequent effects, may be mediated through the intracellular levels of cyclic nucleotides in such a way as to promote the initiation of the mitotic sequence. Oestrogens are thought to oppose these events at an early stage in the sequence, possibly by preventing the initial fluxes of calcium or potassium. A speculative scheme to account for these results is included in the following section.

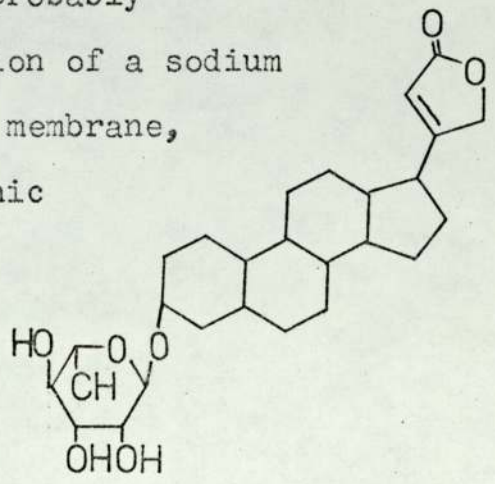



Fig.91: Ouabain

## XV Discussion

If a parallel is to be drawn between the changes in the state of membrane polarisation and mitotic stim--ulation it is necessary for those events which are associated with cell division to be defined and dis--cussed.

The thymic lymphocyte, used in much of this work, is the probable precursor of the T-lymphocytes present in peripheral blood[269]. It is a highly special-ised and responsive cell which plays a crucial role in cell-mediated immunological reactions[84]. One of the principal responses of the cell type is multi--plication of the cell population in response to local stimuli. Thus, the mitogenic stimulation of thymocytes is a manifestation, in vitro, of events which are highly probable in the in vivo situation. The thymo--cyte is very unlike either the blood platelet or the mature erythrocyte in that it is not a 'terminal' cell. It retains the facility for cell division with--out terminal cytodifferentiation. Most of the cells in the culture used in these experiments are likely to be in a resting stage (Go), in which they are involved in neither DNA synthesis or mitosis. There is some controversy over the exact phases of the cell cycle governing cellular replication, and there are no clear rules for the precise description of the various states. Fig.92 shows the'classical' com--partments of a population of proliferating cells. 'S' is the state in which the cells are actively

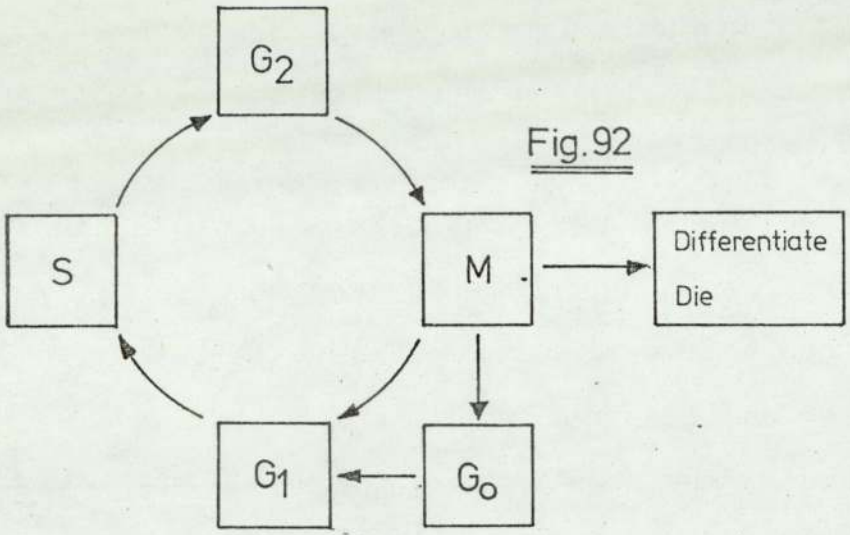



Fig.92

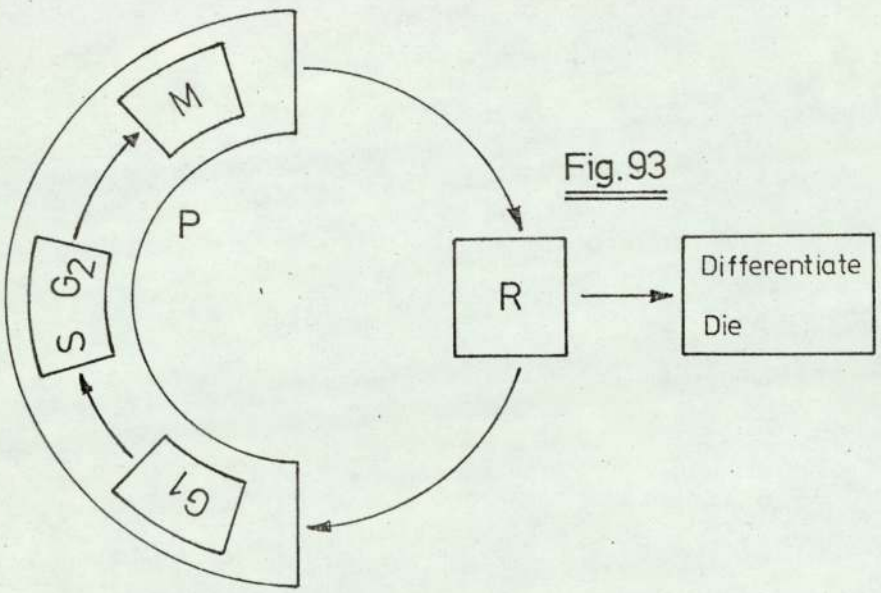



Fig.93

THE CELL CYCLE SCHEMES

synthesising DNA, and mitosis is the active cell division. $G_1$ and $G_2$ are intervals of low apparent activity between the phases. $G_o$ is a resting phase with no precise definition. The cells may leave the cycle, $G_1$-S-$G_2$-M , and enter $G_o$ (from whence there is no return to the cycle). Mitogens, according to this scheme, might act to promote the unscheduled exit of cells from any of the three G phases.

It has been suggested that a modified version of the cell cycle is more suited to the observed data. In this model[386] cells are thought to reside in a resting (R) compartment from which they may be promoted to enter a 'P' compartment which includes $G_1$, S, $G_2$ and M phases. The stages of the P compartment are, according to this scheme, rigidly controlled, with a determined time interval for the passage through the compartment. Mitogenic agents thus can only act in such a way as to promote the exit from the R compartment. The scheme is illustrated in Fig.93.

Replication imposes a heavy demand on the cell--ular economy, because the system must duplicate itself in order to divide. It is probable that a cell does not commit itself to the replication process unless enough supplies of energy are available to permit completion of the process. One can therefore postulate that a cell must have a parameter of its energetic status which would indicate whether cell division was feasible. Such a parameter might be cell volume, or the cytoplasmic concentration of some basic

nutrient such as pyruvate, or even the levels of ATP itself. Adenylate control of metabolism is well established, and the adenylate ratio could exert influence over the rate of cell division. However, it is difficult to see how overall control could be maintained in the mammalian system if the only restriction on cell proliferation was energetic control. For example, it is difficult to imagine how liver cells could be restrained from dividing so well under this system, and yet, although liver cells seldom divide, they all retain the ability to do so, given appropriate conditions. It is far more likely that there exist certain messengers which act as signals for cell division. These might be tissue-specific, and could cause those cells which were energetically competent, and which were also responsive to the messenger, to divide under suitable conditions.

Thus, it seems reasonable to assume that, normally, extracellular nutrients are not growth-limiting in the in vivo situation, and that proli--feration is essentially dependent on other regulators, which might be membrane transport system regulators or specific cell proliferation regulators[18].

In the in vitro situation, more specifically, in thymocyte suspensions, we are faced with a situation in which a proportion of cells can be induced to divide by a number of different agents (see page 150). In the case of, for example, histamine, the exposure

of the cell culture to the mitogen results in only a small percentage increase in the number of cells entering mitosis over a 6 hour period. Presumably all the cells are exposed, to an equal extent, to the mitogen, and so one is posed the problem of why some cells divide whilst others do not. The explan--ation might be that there is a size distribution of cells, and that only those cells over a critical cell volume are capable of cell division, but there is no clear evidence for this. Again, one might postulate that only a certain proportion of the cells had the required $H_1$ receptors. However, this can hardly be an adequate explanation for the small proportion of cells in which cell division is promoted. A23187, a potent mitogen in the presence of calcium, again only stimulates a small number of cells to divide, and surely this ionophore can exert its effect on all the cells in a suspension with more or less equal efficiency.

We are forced to the conclusion that, in the in vitro situation, in the presence of a mitogenic agent, there is a further element of control which governs the cellular behaviour. For reasons which have already been stated, this element is likely to represent the energetic status of the cell. Candidates for this element which have already been mentioned are metabolic intermediates such as pyruvate or acetate, and ATP/ADP,AMP ratios. However,

these are subject to variations within the cell, and their concentrations are held far from static. ATP itself is subject to constant concentration fluctuations which hardly provide a stable basis for control of cell division. A far more likely control agent is cyclic AMP, which is a metabolite of ATP, and which can thus reflect energetic status without being subject to violent oscillations in concentration as a result of cellular metabolic demands. Further, it is known that an increase in intracellular cAMP concentrations can trigger cell division[440].

Whether cAMP is the final messenger of energetic status, or simply an intermediate, is not clear, but it is fair to postulate that the cell is responsive to intracellular concentrations of cAMP; further, that when cAMP concentrations reach a threshold level, cell division is triggered. This threshold will be established at a high level: one which is unlikely to be attained during the normal metabolic sequences, by more than a small number of cells. A mitogenic agent will be one which elevates the intracellular cAMP concentration above this threshold. Parathyroid hormone, vasopressin, prostaglandins ($E_1$), bradykinin, adrenaline and somatotrophin are all mitogens, and are all thought to elevate intra--cellular cAMP levels[37, 244, 308, 438, 441].

The situation appears to be more complex, however, since Morgan[257] has shown that mitogenic

hormone can be separated into two distinct functional groups. Members of the first group (including PTH, acetylcholine, insulin and histamine) require calcium ions to be present if they are to exert their effect; and members of the second group (including glucagon, dopamine and isoprenaline) require magnesium ions. In addition, we have already seen how calcium and magnesium, independently, can act as mitogens in the thymocyte; and how oestradiol and testosterone can inhibit these activities (and the activities of the ion-dependent mitogens); the oestradiol blocking the calcium, and the testosterone blocking the magnesium axes[275]. Further, acetylcholine and insulin have been shown to elevate cGMP levels in cells[136,170,210] Thus it might appear that those mitogens of the calcium axis exerted their effects on the cell via cGMP, and not cAMP. However, one property of cGMP is to inhibit cAMP phosphodiesterase[126], an enzyme responsible for the degradation of cAMP; so elevated cGMP levels could in turn promote elevated cAMP levels, and cAMP would remain the final cellular messenger. In spite of this, the paradox exists that the intracellular cAMP concentration is at its lowest during mitosis[62], is inversely related to the rate of cell growth[302, 303], and is elevated in contact-inhibited cell populations[21,203]. Hait[149] has shown that the abnormal proliferative activity of leukaemic lympho--cytes could be attributable to an increased activity

of one of the multiple forms of cAMP phosphodiesterase which are known to exist[406,414,415] . Further, in almost all cell investigated there has been found to exist a calcium-dependent protein activator of cAMP phosphodiesterase[351,450]. So the rôle of cyclic nuc--leotides is far from clear.

The rôle of ions as mitogens is also unclear. It has been suggested[276] that since calcium stimulates guanyl cyclase activity[150] that cellular cGMP (and ultimately cAMP) levels are moderated by this ion; this being its mode of action in mitotic stimulation. Calcium has been reported to inhibit cAMP-dependent protein kinase activity[213,247], and so it may not simply be the levels of cyclic nucleotides which are important but also their activities. (Protein kinases are believed to mediate the physiological effects of cAMP[212] and cGMP[211] . Calcium can modulate some of the effects of cAMP on cell growth[442] , and is required for some of the effects of cGMP[363] . The fact that cAMP and cGMP can often have antagonistic effects on cell growth[137] has given rise to the so-called 'ying-yang' hypothesis, which appears to be reflected in the membrane polarisation studies (page 151 ). This hypothesis is almost certainly an oversimplification.

The work described here shows that cGMP, besides stimulating mitosis, also triggers a membrane hyper--polarisation. Calcium has a similar effect, and the response can be inhibited by oestradiol. cAMP has

no clear effect on the membrane potential, but magnesium could attenuate the effect of calcium in this respect, and testosterone (which inhibits the mitogens which operate via the magnesium axis) promoted a change in trans-membrane potential opposite to that promoted by oestradiol. There is a remarkable similarity between the hyperpolarisation and the mitotic situations in these respects. The question is, what is the link, if any, between the trans-mem--brane potential changes and the mitotic stimulus?

The importance of divalent cations to the main--tainance of membrane structure has been discussed in section XI . It was stated that addition of, for example, calcium to membrane lipids can promote a phase transition, by decreasing the surface charge of the membrane, which could well lead to an increase in the proportion of the lipids of the membrane existing in the gel phase at a given time. The extent of this transition will depend on the cooperativity of the membrane, but will, in any case, alter the distribution of grain boundaries, and will thus ammend the permeability properties of the membrane. Further, addition of calcium to only one side of a membrane can have even more dramatic effects which radically alter its permeability properties.

As has already been stated, the concentration of calcium in the medium used to support the thymocytes was between 0.6 and 1.2 mM, and the in

vivo plasma ionised calcium concentration is about 1.0 mM. In contrast, the internal calcium concentration is very low (of the order of $10^{-7}$M[43,127]). As a consequence, one might imagine that those (high affinity) binding sites for calcium which exist in the outer lipid palisade (perhaps consisting of phosphatidic acid residues) will be saturated with calcium (and/or magnesium), whereas those in the inner palisade will not. As a consequence, calcium may well exert an effect on the cell, from the out--side, by interacting with (medium-low affinity) sites on the outer palisade, but the effect will be much more dramatic if it is exerted from the inside of the cell[40,340]. This perhaps explains the efficiency of A23187 in promoting mitosis and other effects[118]. It has been shown[339] that a perturbation of the molecular organisation of the surface membranes of mammalian phagocytes leads to significant modi--fications of their metabolism and functions, witnessed by a dramatic rise in cell respiration when calcium ions are allowed to enter the cell via A23187. In addition, A23187/calcium is known to catalyse potassium release from erythrocytes[332,198] and the parotid gland[372]; and also a release of histamine from mast cells[125]. The fact that valinomycin also caused an increase in metabolic activity in leuko--cytes (PMNL)[339], presumably by virtue of a release of potassium from the cell, lends support to the

suggestion that potassium efflux may well be impor--tant in the calcium response. Fig.75 has shown that one of the earliest responses to calcium stimulus in the thymocyte is change in the membrane permea--bility to potassium, this being important in the generation of the membrane potential changes. Lew[225] suggested in 1974 that, since calcium influx into metabolically depleted cells depends on the external potassium concentration, it would seem conceivable that external potassium regulates calcium influx, and thus controls the calcium effect at the inner surface of the plasma membrane.

In general, the permeability of the thymocyte membrane to ionised calcium is very low. Intracellular calcium homeostasis has been demonstrated to be mainly achieved by the action of mitochondria as a buffering and trapping compartment for calcium. The transport of calcium across the plasma membrane has been shown to be only a minor contribution to the adjustment of the internal ionised calcium concen--tration[43,205]. Calcium accumulation by mitochondria is the most rapid and effective energy-linked function[113]. It requires coupled respiration or the hydrolysis of ATP. Mitochondria have a high affinity for calcium, greatly exceeding that for ADP, and calcium is accumulated in preference to the energy being used to phosphorylate ADP.[70] (I should like, at some time in the future, to study this response, and

to find out whether mitochondria can be held in a pseudo 'State 4' condition by limiting calcium conditions rather than limiting ADP. If this were so, then calcium influx into the cell - or any other means of elevating intracellular calcium concentrations would release a block on the oxidative phosphorylation system which could well be the factor governing a cell's ability to divide).

Kolb[205] has shown that external calcium concen--tration markedly affects the membrane permeability to potassium in liver cells. Because the internal calcium concentration is not thought to be of any relevance to this effect, Kolb, in 1976, postulated regulatory sites in the membrane to which either a divalent cation, or two monovalent cations, may bind, with the possibility of an exchange between both states of binding. Further, there are thought to be interactions between the regulatory sites rendering this exchange a cooperative process. The regulatory sites govern the membrane permeability to potassium, with a divalent cation-bound site denoting a 'closed' channel and a double alkali ion-bound site denoting an 'open' one.

McLaughlin had proposed in 1971[261] a mechanism whereby divalent cations exert their effect on ion permeability by changing the Gouy-Chapman potentials of the electrolyte surface layer of the cell membrane, but this was dismissed by Kolb in favour of the regu--latory site ideas.

Neither the results of the work with lympho--cytes reported here, nor those from the (unpublished) work on the effect of calcium on the 5-HT release from platelets, point to any precise mechanism governing the potassium release from the cell which occurs on stimulation with calcium. Clearly, the general statement can be made that elevating the external (or internal) calcium concentrations alters the permeability characteristics of the cell membrane; and this is the first time that the membrane of a cell type such as the lymphocte has been demonstrated to be electrically excitable in this way.

In parenthesis it might be noted that the res--ponse is known to work in reversein some membrane systems, for example, the ciliate protozoan Paramecium has an excitable surface membrane which depolarises in response to a sudden increase in sodium or pot--assium concentrations of the outside medium. An influx of calcium during this depolarisation raises the internal calcium concentration, which in turn causes the cilia to reverse their direction of beating, and the cells to swim backward, away from the high salt concentration[61].

There is no theory, yet, regarding the potassium, and other, ion fluxes in cells other than the trad--itional excitable cells such as neurones. The very intense interest in this field in the last couple of years shows that the topic may well have great general significance.

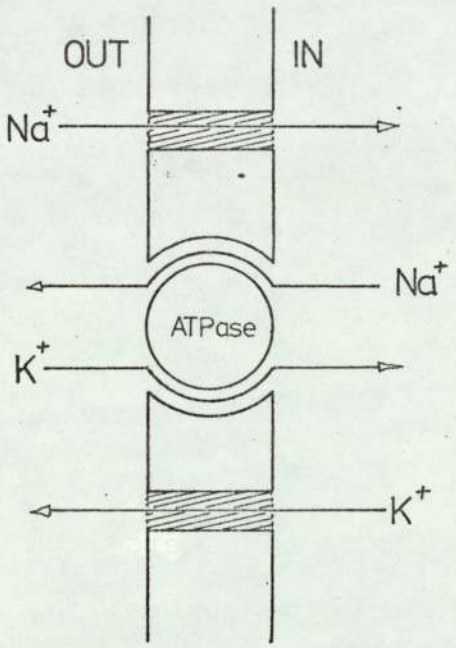



Fig.94:
Sodium/potassium ATPase.
Hatched sectors denote
passive leaks of ions.

The specificity and complexity of the ionic interactions leads one to suspect that the potassium permeability change is the manifestation of an ordered system with functional significance in the intact cell. In contrast to a rigidly coupled pump flow of potassium and sodium ions, a potassium-specific leak mechanism, operating in conjunction with the potassium/sodium pump would permit independent regu-lation of cell volume and potassium concentration (Fig.94). (As was mentioned earlier, cell volume may well be a trigger for cell division; i.e. the attainment of a critical cell volume might well be the required mitotic stimulus which governs cell division). The sodium/potassium pump is an energy-consuming system, and it termed a sodium/potassium ATPase; (ATP phosphohydrolase, EC 3.6.1.3.). It has been demonstrated that insulin stimulates an ATPase activity in membrane fragments from human lymphocytes[145] and also from frog skeletal muscle[131] ; and it was initially thought that modification of the properties of this enzyme by calcium and/or mitogens could explain the membrane hyperpolarisation.

The sodium potassium ATPase is thought to operate according to the scheme shown in Fig.95[338] . The conversion from AP to BP entails a loss of energy, and a change in conformation[317] . Typically, the sub-strate for the enzyme (magnesium-ATP), together with sodium ions, bind to the enzyme at the inner face

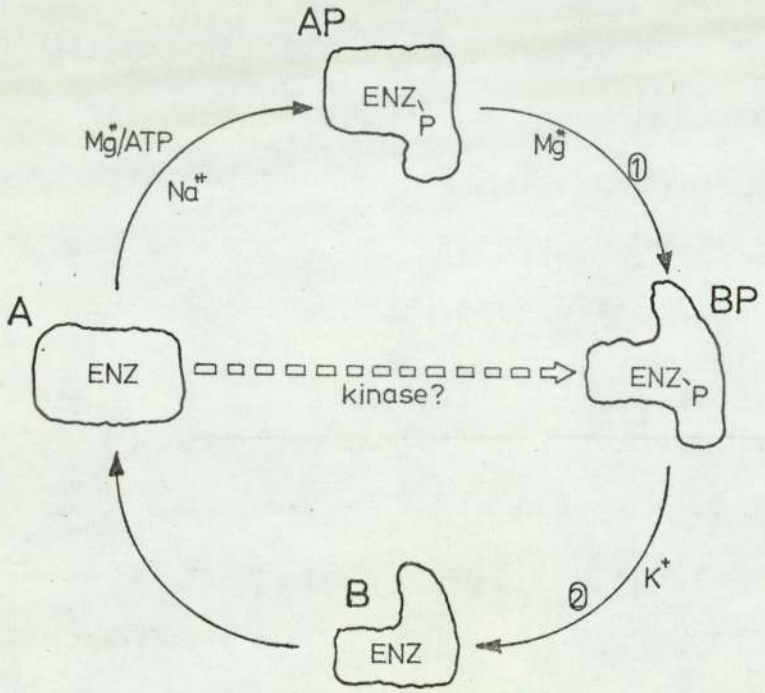



Fig.95:

Conformational changes of the sodium/potassium ATPase.

(1): Inhibited by sulphydryl agents

(and also oligomycin)

(2): Inhibited by ouabain

of the cell membrane. The ATP is hydrolysed, and the enzyme becomes phosphorylated. The phosphorylated protein suffers a change in conformation which allows potassium to bind at the outer face of the membrane, and the resultant effect is ingress of potassium ions, egress of sodium, and hydrolysis of ATP. There is a direct stoichiometry between sodium out and potassium in.

The evidence presented here suggests that calcium stimulates the Na /K ATPase either by a direct action on the protein, or by mechanisms mediated by the lipid components of the membrane. The question is, is the hyperpolarisation secondary to the stimulation of the sodium pump or is it the primary event? The ion flux experiments showed that the movements of potassium out of the cell were much greater than the movements into the cell, and also that potassium ions were involved to a much greater extent than sodium ions, which, given the stoichiometry of the pump, would suggest an alternative mechanism. However, it is feasible that, if the effects of calcium are mediated through cyclic nucleotides (which are known to activate protein kinases) then there could be a direct conversion of A to BP (Fig.95), under the action of a cAMP/cGMP-dependent kinase which would uncouple potassium transport from sodium transport, and promote dramatic ion fluxes. The ATPase and adenyl cyclase enzymes are closely linked[376], and it may

even be that the two functions represent different activities of the same protein. There is certainly a strong probability of the two enzymes (if there are two) colliding, and interacting, in their passage through the lipid matrix (see page 123). The conversion of A to BP would still result in a route which would be inhibited by ouabain; and the primary response of the system would still be an initial influx of potassium into the cell; whereas the results presented here suggest that the hyperpolarisation, although moderated by ouabain ($10^{-4}$M), was not wholly inhibited. Further, the primary response was that of a dramatic potassium efflux.

It is entirely consistent to suggest that, if there is a strong relationship between the activities of the ATPase and cyclase proteins, then this need not be solely a one-way interaction. Agents which block the action of ATPase are also known to cause an increase in cAMP levels[377], and there may be a two-way reciprocal reaction between the two activities. Thus, a scheme can be suggested to explain the actions of calcium on cell proliferation which is at the same time speculative and also responsive to the observed results.

Calcium may inhibit the activity of membrane-bound $Na^+/K^+$-dependent ATPase in one of two ways: Firstly it may bind to (phosphatidyl serine) molecules in the membrane inner or outer palisades so as to

yield solid (gel) aggregates which cause other phospholipids to form fluid clusters[296]. If this should occur at the cytoplasmic surface of the cell, where most of the phosphatidyl serine is located[374,456] it would probably lead to an aggregation of intramembrane proteins[142]. This change could result in a modification of the activities of cyclase/ATPase proteins. Secondly, the ATPase might be stimulated as a result of the sudden loss of cytoplasmic potassium caused by an initial increase in the membrane permeability to that ion, induced by the action of calcium on the lipid phase. It has been stated (page 110) that a change in the proportion of gel phase and liquid crystalline clusters will alter the grain boundary distribution, and thus alter the proportion of transport channels within the membrane.

X537A and A23187 both promote changes in membrane fluidity[8], and Con-A, when it binds to cell membranes, causes a re-distribution of protein sub-units[287,316] which seems to be associated with fluidity changes. There is no evidence for initial opening of calcium channels, or pores, after stimulation by calcium, but the membrane hyperpolarisation initiated by the potassium flux could well promote the displacement of calcium from the cytoplasmic membrane, which would, in turn, induce changes in the organisation of peripheral proteins on the inner face of the membrane. Such proteins have been

suggested to act as regulatory restraints in deter--mining the movement of receptors on the outer face of the plasma membrane[108,184,288]. This effect might well be the basis of the calcium requirement of receptor-mediated mitogens such as Con-A, insulin and histamine.

Oestrogens probably interact with the cell membrane in such a way as to prevent these fluidity changes. Some of the effects of local anaesthetics, such as dibucaine, result from their ability to displace calcium associated with the anionic groups of acidic phospholipids[305], and dibucaine can cer--tainly interfere with the movement of Con-A receptors within the membrane[315]. Many steroids exert an anaes--thetic effect, in spite of the marked difference in the shape of the steroid nucleus. Furthermore, steroids are very lipid-soluble and show some of the non-specific effects of other general anaesthetics[368] for example they protect erythrocytes from lysis[367], presumably by allowing their membranes to expand without fracture[368]. Richards has suggested that specific receptor sites exist for certain steroids such as alphaxalone (Fig.96)[336], but there has been no comparable work with oestrogens.

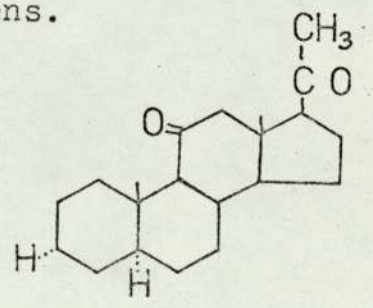


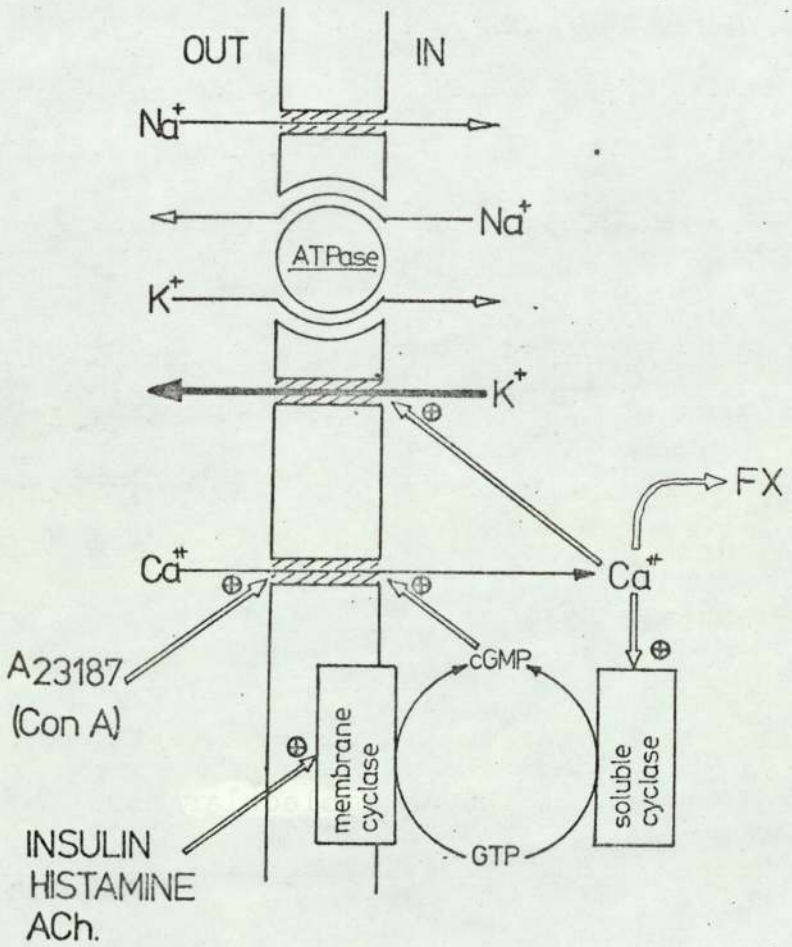

Fig. 96: Alphaxalone

It has been shown[193] that oestradiol-17β floats on the surface of the membrane, (rather like a coin floating on the surface of a brush); and that the membrane acts as a focus for the oestrogen, tending to concentrate the steroid at the lipid/water inter--face. It is clear, however, that some oestrogen will dissolve in the matrix of the lipid, and some cell membranes, such as those of platelets[313] oxidise the oestradiol to oestrone - suggesting more than a casual interaction between the steroid and the membrane.

If the rôle of the membrane interface is to concentrate, orient and even favour the association of molecules of the hormone, and eventually to precipitate the molecules of the hormone on the lipid bilayer[193], we might expect steroid-lipid interactions to take place. From many recent studies on the phospholipid-steroid interaction it has become clear that different steroids have quite different effects on the molecular mobility and functional property of lipid bilayer membranes[166,218,381] Huang[166] has commented on the difference in behaviour between 3a & 3B epimers of sterols, and has suggested that the rigid, planar fused ring system of a molecule such as cholesterol is inserted vertically into the hydrocarbon core of the membrane with the C-3 at the hydrocarbon surface allowing hydrogen-bonding to take place between the 3-hydroxyl group and a phospho-

Fig.97:

Mechanism 1



lipid carbonyl group. Oestrogens suffer no epimeris-
-ation at the 3-position, and the suggestion that
they orientate parallel, rather than perpendicular,
to the plane of the bilayer[193], precludes them from
being involved in quite the way that Huang suggests.
However, the different activities of the methylated
oestrogens, and the importance of the aromatic centre,
indicate that these properties are vital to the spec-
-ific interference of oestrogen with hydrocarbon
packing. Further, the hydrogen-bonded structural
effect of oestrogen may also lead to long-range
effects on the packing density of the hydrocarbon
chains of neighbouring phospholipids; and such changes
in lipid packing would be expected to affect the
permeability properties of the lipid bilayer[381], or
the activities of membrane-associated proteins (such
as guanylate cyclase[141]).

In conclusion, I offer two mechanisms to account
for the observations on calcium, and mitogen, stim-
-ulated hyperpolarisation and mitosis in thymocytes.
Mechanism 1: (Fig.97).: There is a slight leak of
calcium into the cell. In the case of lymphocytes,
the intracellular calcium concentration is kept low
by the concerted actions of the mitochondrial
accumulation of calcium, and the plasma membrane
calcium extrusion pump, the $Ca^{2+}/Mg^{2+}$-ATPase.[358,359] (In the
case of the mature red cell, only the extrusion
pump is available,[358] there being no mitochondria; and

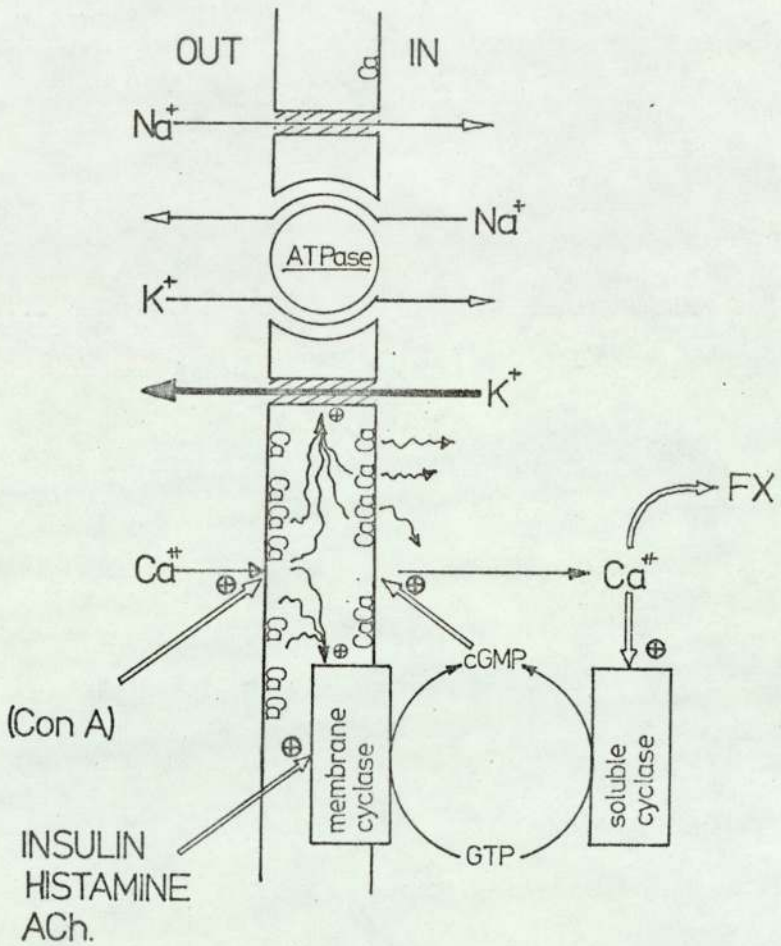
in the case of the platelet there are highly potent calcium-accumulating organelles, very similar to the sarcoplasmic reticulum of smooth muscle). If this leak exceeds a critical value, either because of an increase in the extracellular calcium concentration, or because of an increased permeability to calcium induced by an ionophore or lectin, then a train of events is initiated which results in membrane hyper--polarisation and mitosis. The effect of increased intracellular calcium is to promote an efflux of potassium (because of its effect on the lipid phase proportions) which in turn promotes more rapid calcium influx because of the hyperpolarised state of the membrane. This feed-back is augmented by the action of cGMP, whose concentration is increased because of the stimulatory action of calcium on the soluble guanyl cyclase[141] . The final effect of this positive feed-back is to elevate, grossly, the intracellular calcium concentration. This can now have the effect a) of dissociating particular classes of histone from the nuclear chromatin, thus de-rep--ressing a portion of the genome; b) of modifying the action of cellular enzymes which have a sensi--tivity to calcium (such as cAMP phosphodiesterase) or c) of stimulating oxidative phosphorylation, which will result in an increase of energy (ATP) levels in the cell, and act as the mitotic trigger (see page 158). In this scheme, agents such as histamine

and insulin would exert their effect by binding to membrane receptor proteins and modifying their shape. This conformational change would, by an allosteric effect, or via an intermediary transducer protein, cause a change in the activity of a membrane-bound cyclase enzyme (for example guanyl cyclase[141]), resulting in elevated cGMP levels which promote a secondary influx of calcium into the cell. According to this scheme, oestradiol would act in such a way as to inhibit these calcium transients, and thus abolish the effect of the stimulus.[273] Agents such as caffeine and rayanodine, which also abolish calcium transients[224], do so by interfering with the intra--cellular levels of cyclic nucleotides, and their effects can be predicted with this model. Similarly, ouabain should have little effect on these responses, because they do not depend, primarily, on potassium movements across the plasma membrane. Sodium azide, however, would be predicted to be a potent mitogen because of its stimulation of guanylate cyclase. Work with these agents, and also agents such as tetrodotoxin, veratridine, dipyridamole etc. would help to establish just how valid this interpretation is.

It has proved notoriously difficult to determine whether calcium actually enters the cell after stimulation by, for example, Con-A[127]. It is hard to disentangle the calcium binding to the outer face

Fig.98:

Mechanism 2



of the cell membrane, and that binding to inner structures. It is even more difficult to establish whether the intra-cellular ionised calcium levels change after stimulation[43]. To allow for the possibility that calcium does not enter the cell in order to exert its effect, the preceding model must be ammended.

Mechanism 2: (Fig.98): When the extracellular concen--tration of calcium is raised there is a greater extent of calcium binding to low affinity sites of the lipid palisade. This calcium binding, to the outer face of the cytoplasmic membrane, causes phase changes in the lipid matrix which, again, cause pools of gel and liquid crystalline lipids to rearrange. This rearrangement has 3 effects: a) it alters the permeability of the membrane to other ions such as potassium, thus promoting a potassium efflux, count--eracted by the actions of a sodium/potassium ATPase; which, in turn, results in a sodium efflux, and mem--brane hyperpolarisation; b) it modifies the activity of m mbranous cyclase enzymes, promoting an elevated cyclic nucleotide level within the cell; or c) it permits the movement of membrane-bound receptors for agents such as insulin and Con-A. This movement is most obvious in the case of Con-A, where receptors clump together in large clusters[315]. A framework of proteins on the inner membrane face exerting trans-membrane control of the topography of receptor moieties on the outer membrane face could be linked, as in the

case of spectrin, to acidic membrane lipids by calcium. This type of organisation could provide a mechanism for changes in the mechanical properties of the plasma membrane (and hence the activities of membrane proteins) regulated by calcium, which would not require constant or extensive changes in the overall fluidity of the bulk lipid matrix of the membrane. The result of the movement of these proteins could be that a previously unresponsive cell becomes responsive to a mitogen in the presence of calcium. Oestradiol would be exp--ected to interact with the membrane lipids in such a way as to stabilise the membrane against the calcium triggered changes. The receptor-mediated mitogens would be inoperative in the absence of calcium, and in the presence of oestrogen. In this scheme, the rôle played by the potassium release is not clear. It may be fortuitous, or it may be intimately involved with the potentiation of the calcium effects by promoting the further release of calcium from binding sites on the inner face of the membrane palisade.

To believe is difficult. Not to believe is impossible.

Victor Hugo.

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'The best ornament of all times is neither original nor copied: it must recognise tradition, and add something which shall be the tradition of the future' (W.R. Lethaby)


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