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THE ENCAPSULATION OF PHARMACOLOGICALLY ACTIVE 
PEPTIDES INTO INTACT ERYTHROCYTES.

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The Encapsulation of Pharmacologically active peptides into intact Erythrocytes

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SUMMARY

The encapsulation of the anti-sickling dipeptide, L-Lysyl-L-Phenylalanine (Lys-Phe), and the anti-secretory peptide, urogastrone, into intact rat erythrocytes was investigated.

The survival of resealed carrier erythrocytes was monitored in the circulation by cell counts after labelling with fluorescein isothiocyanate. These cells were found to survive for their full circulating lifetime of about 40 to 45 days.

Carbon-14 labelled Lys-Phe was synthesized as a means of assaying the amount encapsulated inside the cells. Urogastrone was labelled with 125-iodine to produce a tracer solution for assay purposes. Under optimal conditions, it was found that the maximum amount of drug encapsulated was achieved just prior to the point of haemolysis. Loading values of between 14 and 20% were obtained for the two peptides, as determined by radioactivity measurements.

The release of the peptide from erythrocytes was tested in vitro using dialysis sacs. It was found that in both cases, diffusion of the peptide was considerably slower when encapsulated.

The method of encapsulation was compared to an alternative method of entrapment using an antibiotic, amphotericin-B, to facilitate the entry of Lys-Phe. The latter method gave lower loading values and poor survival times of the carrier cells in vivo. The rate of diffusion of the dipeptide from the erythrocytes was also greater.

Lys-Phe, encapsulated into intact SS-erythrocytes, exhibited a marked inhibition in sickling after deoxygenation.

Encapsulated urogastrone was not as effective in inhibiting carbachol-induced gastric acid secretion in the perfused rat stomach preparation, when compared with the free peptide. The duration of inhibition, however, was significantly longer with encapsulated urogastrone.

Encapsulated preparations were stored on either gelatin or agarose gels as a means of prolonging the storage time. Under optimal conditions the preparations were viable for up to 21 days but the lifespan in vivo of these cells was shortened.

KEYWORDS

ANTI-SICKLING  ANTI-SECRETORY  ENCAPSULATION
ERYTHROCYTE  PEPTIDE
ACKNOWLEDGMENT.

I would like to specially thank my supervisor Dr. David A. Lewis who advised and guided me throughout the project. Thanks are due also to Dr. H.O. Alpar, Dr. C. Haigh, Dr. R. Patel, Dr. A. Kafy and Dr. W. Field.

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INTRODUCTION.

1. ENCAPSULATION INTO ERYTHROCYTES.

1.1. INTRODUCTION.

There are two desirable properties for a drug carrier: to selectively direct a drug to a target tissue and to protect the drug from premature biodegradation. Several advantages stem from selective targeting including delivery of localized high drug concentrations and savings on drugs in limited supply. The protection of a drug from biodegradation is particularly important when proteins are being administered or when immunogenic drugs are used. In addition, the carrier must be non-immunogenic and biodegradable.

Erythrocytes have many of the attributes of the ideal carrier. Since the patient's own erythrocytes may be used, there is no danger of adverse effects from foreign substances used to construct the carrier membrane. Not only is the erythrocyte biodegradable, but degradation is accompanied by the same cells and in the same manner as is employed for normal erythrocytes.
It seems clear that erythrocyte carriers can circulate nearly as long as normal erythrocytes (120 day overall lifespan in humans). This extended period of circulation would allow enzymatic reactions to be carried out within the blood and would allow the erythrocyte carrier to be used as a storage depot for sustained release of drugs over a substantial time interval.

Alternatively, modifications of the erythrocyte will activate it for rapid destruction in the reticuloendothelial system, allowing entrapped substances to be delivered to lysosomes or phagocytic cells.
1.2. **THE RED CELL MEMBRANE AND ITS CYTOSKLETON.**

1.2.1. **PHYSICAL PROPERTIES OF THE RED CELL.**

The peculiar fascination that the red blood cell has for so long exercised over biophysicists and physiologists observes from the unique mechanical properties that allow it to endure stresses, to which it is subjected during the several hundred miles that it travels in the course of its 120 day lifespan. The familiar biconcave discoid shape has evolved to meet the requirement for gross elastic deformation under the influence of high shearing stress. The cell must be capable of squeezing through capillaries much narrower than its own diameter and reverting rapidly to the discoid state when it emerges.

In one sense the red cell membrane behaves as though it were a solid, for it is practically inextensible in respect of surface area. By contrast, linear deformation occurs with great ease. The virtue of the discoid shape, thus lies in its capacity to undergo gross deformation with no significant change in surface area; a spherical cell, for which the area/volume ratio is already minimal, would be maximally resistant to shape deformations. It is now regarded as certain that the cells owes the preservation of its integrity, its discoid shape and its elasticity alike to a filamentous structure, covering the cytoplasmic surface of the membrane (Gratzer, 1981).
There is abundant evidence for this conclusion. For example, when red cell ghosts are extracted with media of low ionic strength the protein complex is dissociated, and disintegration of the membrane into the vesicles rapidly occurs. Much of the same occurs if the proteins are destroyed by proteolysis (Lux et al., 1979). The cytoskeleton appears also to be the agency through which the cell exercises a number of biologically important functions, involving for instance the passage of signals across the membrane.
1.22. **METABOLIC REGULATION OF CELL SHAPE.**

Nakao et al. (1960), first recognized that metabolic depletion of the red cell is accompanied by a succession of shape changes. Thus when cells are incubated in the absence of glucose, the intracellular ATP is consumed, and at the same time crenations appear on the membrane surface. The cell then begins to lose its discoid profile and becomes spherically symmetrical though still crenated (type III echinocytes). The protuberances become progressively more elongated, form necks and eventually separate as vesicles or microspheres leaving a smooth spherocyte. Up to the echinocyte, these changes are reversible on addition of metabolites to the medium so as to recharge the intracellular ATP pool.

To be sure, metabolic depletion is by no means the only way in which cell shape changes may be provoked. They can be caused by most forms of physical abuse, and most of all by lipophilic drugs or other molecules that partition into the membrane. Two types of shape transformation can be recognized: in general (Deuticke, 1968), anionic solutes generate echinocytes, with rather large protuberances distributed over the cell surface, and cationic species give rise to stomatocytes, or smooth cup shapes.
Exposure of the cell to two ligands, one of either type, can lead to a cancellation of the two effects, with retention of the discoid shape. Anionic ligands are found to interdigitate mainly in the outer, and cationic ligands in the inner leaflet of the membrane.

1.23. RECEPTOR EVENTS.

The human red cell interacts with many biologically active substances in the nanomolecular concentration range. It possesses binding binding sites for insulin (Herzberg et al., 1980), growth hormone (Rubin et al., 1973) and prostaglandins (Kury and McConnell, 1975).

The first direct evidence of coupling between surface components and the cytoskeleton came from Nicolson and Painter (1973), who found that anti-spectrin antibodies sealed into ghosts caused redistribution of heavy-atom electron microscope markers attached to the saccharide residues of the glycoproteins on the outside; Ji and Nicolson (1974), also reported that when lectins were used to cross-link the external saccharides, chemical cross-linking of cytoskeletal proteins on the inside was facilitated.
1.24. CYTOSKELETAL CONSTITUENTS.

Electrophoretic analysis in sodium dodecyl sulphate/polyacrylamide gels of the total membrane protein reveals the presence of a large number of components. Four of these make up the cytoskeleton. In the standard designation of Fairbanks et al. (1971) (numbering from the origin of migration) these are bands 1 and 2 (which together make up spectrin) 4.1 and 5, which is actin. (A very minor component 4.9 is also present). Spectrin and to a lesser extent the other proteins, are released from the membrane into media of low ionic strength.

Depending on the condition of extraction, the spectrin is recovered either as a dimer, two chains of molecular weight about 240,000 and 220,000 or as a tetramer, consisting of two such dimers associated end-on. It is a markedly acidic protein, and becomes insoluble in the vicinity of its isoelectric point (between about pH 5 and 3). The two polypeptide chains differ from one another in sequence and in certain physical and functional properties (Calvert et al., 1980).
The 4.1 protein is an apparently globular species of subunit unit mol.wt about 80,000. It is probably dimeric in the membrane. The actin (mol.wt 42,000) is of the beta-type. The minor component, protein 4.9, co-purifies with the cytoskeleton, has a subunit mol.wt of about 60,000, and is in other respects uncharacterized.

1.25. TRANSPORT IN ERYTHROCYTES.

The erythrocyte has a high internal potassium concentration and a low internal sodium concentration. This difference is reversed in plasma. In the presence of passive diffusion, the concentration gradient is maintained by the ouabain sensitive enzyme, Na+,K+ ATPase which transports three sodium ions out of the cell for every two potassium ions brought into the cell. Calcium and Magnesium ions are also actively transported.

The active transport system is dependant on ATP generated by glycolysis hence glucose metabolism is important in maintaining sodium and potassium gradients. The system is also closely involved in maintenance of cell volume.
When active cation transport is paralysed, the cells accumulate sodium and water until a critical volume is reached and haemolysis then occurs with a discharge of cellular contents into the surrounding space.

The main erythrocyte anions are chloride and bicarbonate. The erythrocyte membrane is very permeable to these ions compared with its permeability to sodium and potassium. Other ions to which the membrane is permeable include phosphate, sulphate, and some organic anions including pyruvate (Rice and Steck, 1976).

Passive transport of water into erythrocytes is very rapid. Glucose, also enters more rapidly than it is metabolised so that the rate of entry does not limit the rate of glycolysis. Other substances such as sugars and amino acids enter the cell at varying rates (Winter and Christiansen, 1964).
FIG. 1. ORGANIZATION OF THE CYTOSKELETON: SCHEMATIC DEPICTION OF RED CELL MEMBRANE ORGANIZATION.
1.3. ENCAPSULATION TECHNIQUES.

The majority of encapsulation techniques involving erythrocytes employ hypotonic lysis of the cells followed by restoration of tonicity which reseals the cells. When drugs were included in the hypotonic solutions, they were in part, encapsulated by the reformed cells. Ihler et al. (1973), demonstrated that enzymes could be trapped into erythrocytes by rapid haemolysis of the cells with several volumes of distilled water containing the substance to be trapped. Under hypotonic conditions the cells swelled rapidly and large holes or "tears" opened in the membrane enabling the drug to be encapsulated into the cells. The cells were reformed by restoring tonicity and stabilized by annealing for some time at 37°C. Most of the cytoplasmic constituents were lost in these rapid swelling techniques and the reformed cells contained very low amounts of the drug in solution. If the loaded cells were returned to the circulation they were rapidly removed by the reticuloendothelial system.
Presently there are three methods employed which give
good survival characteristics when the cells are returned to
the circulation. These methods can be described as: a)
preswell dilution techniques; b) dialysis techniques; or c)
dielectric breakdown of the erythrocytes.

A. PRESWELL DILUTION TECHNIQUES.
This technique was first described by Rechsteiner (1975). The
erythrocytes were first swelled without lysis by placing
them in a slightly hypotonic solution. The swollen cells were
then recovered by centrifugation at low g and the cells then
taken to the point of lysis by the addition of relatively
small volumes of water.
This gentle swelling of the cells resulted in a good
retention of cytoplasmic constituents and improved survival
times in vivo (Humphreys and Ihler, 1981).

This technique has been recently modified (Pitt et
al., 1983a), and excellent survival times for drug-loaded
erythrocytes in rats, mice, guinea-pigs and rabbits have been
obtained. This procedure was used through this present work
and is described in the method's section. The erythrocytes
are exposed to the minimum hypotonic stress which allows the
cells to become permeable to the substance to be
encapsulated.
To achieve this, the cells are first swollen to a sub-permeable, pre-haemolytic state, followed by a layer of haemolysate, which provides a barrier between the cells and the environment. After which, comes the addition of several small volumes of water or drug solution to the cells. The recognition of the point of the haemolysis is thus vital to avoid unnecessary cell damage caused by over dilution. The cells are then resealed by the addition of calculated volume of hypertonic solution.

Using this method, Pitt et al. (1983a) have successfully encapsulated cortisol-21-phosphate, methotrexate, cyclophosphamide and alpha-1-antitrypsin. Even more recently, Alpar and Lewis (1985), have used this gentle loading technique to encapsulate the antileukaemic drug, L-asparaginase, into tumour bearing mice.
B. DIALYSIS TECHNIQUES.

Dialysis was first employed by Klibensky (1957). In this technique the packed cells were placed in dialysis tubes which were immersed in hypotonic media. The use of dialysis tubes resulted in the retention of cytoplasmic constituents in the vicinity of the erythrocytes reducing the loss of cytoplasmic constituents when the cells were resealed (DeLoach and Ihler, 1977). Human erythrocytes at haematocrit values of 70-80 were dialysed against 1-2 litres of distilled water for two hours at room temperature. At 5-10 minute intervals the dialysis tubes were removed and their contents gently mixed by massage.

After two hours the cells were resealed by restoring tonicity. Substances to be encapsulated were added to the dialysing medium and encapsulation loadings of 30-45% of the materials added have been reported (Ihler, 1983). DeLoach et al. (1980), developed an encapsulation dialyzer based on this procedure which could load up to 150 ml of packed cells in one run.
C. DIELECTRIC BREAKDOWN.

Many cells, including erythrocytes, when subjected to intense electric fields form pores in their membrane prior to complete lysis. Kinosita and Tsong (1978), first used this method of dielectric breakdown as a means to load erythrocytes. Using mouse or human erythrocytes the cells were subjected to electric pulses in (14C)sucrose solutions. It was found that the cells incorporated sucrose when the pulse duration exceeded 20 micro-secs at voltages of 1-2kv/cm. Excellent survival times for the loaded cells were found in vivo (mice) provided that the pulse duration did not exceed 160 micro-secs. In this method the pores remained open at low temperatures but closed completely when the cells were annealed at 37°C.

High molecular weight substances such as urease have been encapsulated by this method (Zimmermann et al., 1976). A major disadvantage of this method was that expensive equipment was required and the method had no obvious advantages over the simple dialysis of preswelling techniques.
D. POLYENE ANTIBIOTICS.

Polyene antibiotics such as amphotericin-B damage microorganisms by increasing the permeability of their membranes to metabolites and ions (Deuticke et al., 1973). This property was exploited with erythrocytes when amphotericin-B was used to load erythrocytes with the anti-leukaemic drug daunomycin (Kitao and Hattori, 1980). Daunomycin added exogenously is impermeable to the red cell membrane, however, when the cells were incubated with a low concentration of amphotericin-B, daunomycin was entrapped in the red cells without haemolysis or alteration in the chemical parameters of the erythrocytes. This preparation was used with marginal success to treat leukaemic mice.
1.4. **SURVIVAL OF RESEALED ERYTHROCYTES IN VIVO.**

Erythrocytes osmotically lysed by dilution have variable survival qualities when reinfused back into experimental animals. Thorpe et al. (1974), for example, found that their erythrocytes were removed rather quickly from the circulation. Most investigators report the initial rapid loss of part (20-80%) of the infused erythrocytes and a much slower rate of disappearance for the remainder. Resealed rabbit erythrocytes survived as well as normal erythrocytes in short term experiments (DeLoach et al., 1977), and chicken erythrocytes had a half-survival of 5 days as compared to 11 days for a comparably handled control. Updike et al. (1976), reported that both enzyme-containing and control erythrocytes decreased with first order kinetics with a half-life of about 8 days in monkeys. Alpar and Lewis (1985) have recently showed that erythrocytes loaded with asparaginase had excellent survival characteristics in two different species, C3H mice and Wistar rats. The cell survival half-life span (t0.5) values were 9-10 days for Wistar rats and 14 days in C3H mice.
Using either the preswell dilution technique or the dialysis technique, mouse erythrocytes containing a bacterial virus, $\Phi X174$, survived with a 4-5 day half-life, although 40-80% of the erythrocytes were initially removed very quickly (Humphreys and Ihler, 1981). DeLoach et al. (1981), have shown that bovine and canine erythrocytes loaded by dialysis had extended survival times in vivo.

Kinosita and Tsong (1978), demonstrated that erythrocytes, carrying $^{14}$C-sucrose entrapped by their electric field procedure, survived in mice with virtually no alteration in kinetics for a 30 day period. Between 78 and 98% of the cells survived, depending on the duration of the electrical pulse. The fact that macromolecules are not lost and the absence of gross damage to the erythrocyte membrane probably accounts for the prolonged survival observed. Their procedure is probably superior to all others for the direct entrapment of drugs in erythrocytes intended to survive normally.

In summary, it seems that there is usually a rapid loss of part of the erythrocyte population. Part of this loss is probably associated with removal and manipulation of the erythrocytes in vitro since such losses are observed with normal erythrocytes removed for $^{51}$Cr-labelling and then reinfused. Part of the erythrocyte population is also damaged by the lysis procedure.
This damage seems to be minimized by protocols that retain the erythrocyte metabolism in as normal a state as possible. Important parameters include preservation of enzymes and cytoplasmic constituents and inclusion of substrates needed for ATP generation, including glucose, adenosine and phosphate. Erythrocytes which survive the period of rapid loss, presumably those with the least damage and most normal metabolism, nevertheless seem to be removed somewhat more rapidly than normal erythrocytes.

Current research on erythrocyte aging has implicated a physiologic autoantibody in the removal of normal erythrocytes (Kay, 1981). Increasing amounts of the antigen are found on cells as they age and it is possible that hypo-osmotic lysis might accelerate formation of this antigen.
1.5. APPLICATIONS OF ERYTHROCYTES AS CIRCULATING CARRIERS.

Marsden and Ostling (1959) demonstrated that high molecular weight compounds could be encapsulated in erythrocyte ghosts by using dextrans over a molecular weight range from 10,000 to 250,000 as the encapsulated material. Ihler et al. (1973) applied this technique in the replacement of enzymes. Rat kidney beta-galactosidase and beta-glucosidase were encapsulated into human erythrocytes and showed that the encapsulated enzymes retained their activity inside the cell.

Attempts have been made to treat Gaucher's disease with glucocerebrosidase encapsulated in erythrocytes (Beutler et al., 1977). The experiments were inconclusive but the loaded cells were found to survive for up to 10 days in treated patients. No abnormal reactions were found towards the cells found in the patients and their blood counts, blood pressure and renal function remained normal throughout the treatments.

Green et al. (1980) encapsulated the drug desferrioxamine in erythrocytes to treat patients with excess stores of iron. This drug is rapidly cleared from the circulation and the substance was loaded into erythrocytes in an attempt to slow down its clearance. The encapsulated drug significantly increased the rate of clearance of iron.
Adriaenssens et al. (1976) took cells from patients with familial hyperargininaemia (i.e. arginine-deficient erythrocytes), and were used to encapsulate bovine liver arginase by hypotonic lysis and the cells were resealed by annealing at 37°C after isotonicity was restored. The cells were maintained at 4°C in vitro in acid-citrate-glucose medium for 12 days and throughout that period they maintained urea production. Non-treated arginase-deficient cells from the same patients produced only traces of urea.

A possible application using drug encapsulated erythrocytes is that drugs could be targeted to the liver or spleen. DeLoach and Barton (1981), found that macrophages grown in culture were able to take up glutaraldehyde-treated erythrocytes loaded with beta-galactosidase, or alkaline phosphatase, into phagolysosomes and that enzymes remained active for up to 30 hours in culture. When returned to the circulation, erythrocytes, treated with the minimum of glutaraldehyde were removed by the spleen, but erythrocytes treated with higher concentrations of glutaraldehyde were removed by the liver.
The anti-leukaemic drug, L-asparaginase, has been encapsulated in erythrocytes and tested in several animal species. Updike et al. (1976), found that plasma levels of asparagine were significantly depressed in monkeys; and using the same system in mice, Alpar and Lewis (1983), completely removed asparagine from the plasma in vivo over a 14-day period.

Subsequently the same workers have reported that the encapsulated enzyme was able to "cure" mice implanted with the 6C3HED tumour whereas the same amount of free enzyme did not (Alpar and Lewis, 1985).

The encapsulation of low molecular weight material has produced variable results. Pitt et al. (1983a), encapsulated corticosteroid phosphate esters which were unable to escape from the cells. The action of endogenous phosphatases in the erythrocyte hydrolyzed the ester allowing the free steroid to diffuse through the membrane and maintain useful therapeutic blood levels of steroid for at least 10 days. Encapsulated corticosteroid phosphate esters were found to be superior to free steroid phosphates administered intravenously in the treatment of adjuvant induced arthritis in the rat (Pitt et al., 1983b).
Goldsmith et al. (1979), demonstrated that human Factor IX activity could be incorporated into erythrocytes. They suggested that entrapped Factor IX might be useful in the therapy of patients with severe haemophilia B, presumably relying on a low level of intravascular haemolysis to permit the protein to reach the plasma.

1.6. FUSION OF ERYTHROCYTE CARRIERS WITH VIABLE CELLS.

Several groups (Schlegel and Rechsteiner, 1975; Loyter et al., 1975), have reported the successful fusion of erythrocytes carrying various substances, with other cell types. Fusion can be accomplished either using Sendai virus or polyethylene glycol. Schlegel and Rechsteiner (1975), introduced 125I-BSA and thymidine kinase (TK) into 3T3-4E cells (a TK-deficient mouse cell line). They demonstrated a physical transfer of protein by autoradiography. Small RNA molecules have also been encapsulated in erythrocytes and transferred by fusion. Capecchi et al. (1977), isolated an HGPRT cell line from mouse L cells which contained a UAA nonsense mutation and showed this mutation could be suppressed by the appropriate E.coli suppressor tRNA introduced via fusion. They also showed that the tRNA activity was not destroyed within the erythrocytes.
In principle, erythrocytes loaded with DNA could be used as an efficient method to introduce genetic material into other cells but there have been no published reports of high molecular weight DNA being encapsulated in erythrocytes. Rechsteiner (1976), reported failure to load with rRNA or DNA. Ihler (1983), suggested that free high molecular weight DNA could enter the erythrocyte if it were condensed and neutralized, for example, with polylysine or histones or even precipitated with calcium phosphate.
1.7. PRESERVATION AND STORAGE OF RED CELLS.

Very few reports have commented on the storage of materials encapsulated in erythrocytes. Most workers appear to have encapsulated their preparations immediately before returning them to the circulation. The well-known techniques available for the storage of erythrocytes used for transfusions are equally applicable to erythrocytes loaded as carriers with drugs or enzymes. Hubbard et al. (1979) found that ATP concentrations were 63% of normal in mouse erythrocytes that were prepared by a dialysis procedure (Sprandel et al., 1979), employing buffers containing glucose and inosine. These workers also reported that if buffers contained glucose, adenosine, phosphate buffer and 4mM MgCl₂, ATP concentrations were restored to normal levels.

The role of nucleotides, especially of adenine nucleotides, in the maintenance of structure and function of red cells has been studied extensively by Strauss and Verdier (1980), and it was concluded that an improved maintenance of viability can be obtained with the addition of adenine, guanosine and inorganic phosphate to stored red cells.
When Green et al. (1980), used desferrioxamine encapsulated in erythrocyte ghosts to treat patients with excess iron stores, blood was taken from patients directly into blood bags. The whole procedure including the removal of blood and encapsulation took 4-5 hours, however, using this method, compatibility and sterility problems were minimal.
2. **SICKLE CELL ANAEMIA.**

2.1. **EPIDEMIOLOGY AND GENETICS.**

Sickle cell disease may be defined as the presence in a person of two abnormal genes for haemoglobin, at least one of which is the sickle cell gene. When both genes are the sickle cell gene, the person is described as suffering from sickle cell anaemia. A person who has a normal gene for adult haemoglobin and a gene for HbS is termed a sickle cell trait carrier (Acquaye et al., 1981).

Its major manifestations are a chronic haemolytic anaemia and vaso-occlusive crises that causes severe pain as well as long term and widespread organ damage, notably the spleen, lungs, liver and parts of the central nervous system. In addition, there are systemic effects from sickle cell disease such as increased susceptibility to infections and impaired growth and development (Acquaye et al., 1981).

The disease has its highest incidence in black Africans and Afro-Americans. It is also found in Mediterranean countries such as Greece, Italy and Israel, as well as in Saudi Arabia and India (Dean and Schechter, 1978). Africans have known of the disease for generations, and it has been traced back as far as 1670 in one family belonging to the Krobo tribe in Ghana (Kontey-Ahulu, 1974).
In the United States, Herrick reported in 1910 on a West Indian student who had "a case of severe anaemia... (with) ... peculiar elongated and sickle-shaped red blood corpuscles". When the third case of sickle-cell anaemia was reported in 1917 the "possibility of an inherited anomaly suggested itself" when abnormal cells were noted in a parent's blood (Emmel, 1917). However, it was not until the late 1940's that Neel demonstrated that sickle cell anaemia was transmitted as a recessive gene.

Because of the high morbidity and mortality associated with the homozygous expression of the sickle gene (the SS genotype), the frequency of the mutant gene would be expected to decline in any gene pool. This decline has not been observed in Africa, however in the United States this gene frequency is expected to decline, although slowly over many generations (Rucknagel, 1974).

Raper (1949) was the first to suggest that the heterozygous condition may afford "protection from parasites". There is a great deal of clinical evidence to support this concept (particularly for Plasmodium falciparum), (Neel, 1944), but the mechanism of protection is unclear (Friedman, 1978).
2.2. HISTORICAL PERSPECTIVES.

Although the sickling process was initially described by Herrick in 1910, it was not until 1923 that Huck noted that sickling was reversible. In 1927, Hahn and Gillespie demonstrated the dependance of sickling on the oxygen tension, and so attributed the defect to the intracellular haemoglobin and not to the red blood cell itself.

In 1939, Diggs and Bibb noticed that some cells were irreversibly sickled even after reoxygenation. One year later Ham and Castle proposed a comprehensive explanation of the pathophysiology of the sickling process. As red cells sickle in the peripheral blood, there is an increase in the bulk viscosity of blood, leading to delayed passage through capillaries, decreasing further the oxygen tension and causing more sickling. Anything that would increase blood viscosity would exacerbate the sickling of cells. This theory has predominated for more than 35 years, even though it has never been well substantiated by experimental evidence. It is consistent with the clinical manifestations of sickle cell anaemia and has been a useful model for further research.
In 1946, Sherman reported that sickle cells (but not normal cells) exhibit optical birefringence when they are deoxygenated. This was the first evidence that sickle haemoglobin had an ordered structure in the cell after deoxygenation. In 1949, Pauling demonstrated a difference in electrophoretic mobility between sickle and normal haemoglobin and attributed this phenomenon to a charge change in the globin chain.

In 1956, Ingam showed that the change was due to the substitution of a valine residue for the normal glutamic acid at the sixth position of each beta-globin chain of the haemoglobin molecule. Although the defect in sickle haemoglobin had been identified, it was still not understood how this single amino acid substitution could cause haemoglobin to aggregate, cause red cells to sickle and lead to all the clinical manifestations of sickle cell anaemia.
2.3 SICKLE HAEMOGLOBIN.

All adult haemoglobins are composed of four polypeptide chains, form the haemoglobin tetramer (Konigsberg et al., 1963): two alpha chains, each with 141 amino acids, and two non-alpha chains, each with 146 amino acids (Konigsberg, 1961). Each chain surrounds an iron porphyrin molecule (haem), which contains a central iron atom to which oxygen is reversibly bound (Baldwin, 1975). In the adult, 96-97 per cent of the non-alpha chains are beta chains that associate with the alpha chains to form the A haemoglobin tetramer (alpha 2 beta2). A much smaller percentage of non-alpha chains consist of delta chains which differ from beta chains at 10 amino acid residues, or gamma chains, which differ from beta chains at 39 residues. The delta and gamma chains complex with alpha chains to form the A2 haemoglobin tetramer (alpha2 delta2) and the F (or foetal) haemoglobin tetramer (alpha2 gamma2), respectively (Schroeder et al., 1963).

To account for the normal adult adult haemoglobins there must be at least four different genetic loci encoding the sequences of the globin chains (Schroeder et al., 1968). Each known haemoglobin mutant characterized by a single amino acid change can be accounted for by a single point mutation in the DNA encoding the beta globin sequence.
In sickle haemoglobin, a single point mutation in the DNA codon for glutamic acid appears to result in a base triplet that is transcribed into a message RNA (mRNA), which codes for valine. It is assumed that the globin chain is synthesized with use of the abnormal mRNA template, and the abnormal beta globin chain then complexes with alpha chains to form the sickle haemoglobin tetramer (alpha2 beta2s), which is called haemoglobin S (Marotta et al., 1977).

It has been known for some time that the overall structure and function of HbS is similar to that of haemoglobin A (HbA), for example, the solubilities of oxygenated HbS and HbA are similar and that their oxygen affinities are equal in dilute solution (Perutz and Mitchison, 1950). In dilute solution the physical properties of deoxygenated HbS and HbA are also very similar. However, concentrated deoxygenated HbS differs markedly from deoxygenated HbA and this is the physicochemical basis of gelation and sickling. When HbS is deoxygenated in vitro under near physiologic conditions it becomes relatively insoluble as compared to HbA and aggregates into long polymers (Finch et al., 1973).
The most physiologically important effector of the polymerization of HbS is oxygen. Only the deoxygenated confirmation of HbS can gel; none of the biligand states of the HbS molecule (i.e. oxy, met, carbomonoxy etc.), will normally aggregate (Briehl and Ewert, 1974). It has been postulated that deoxygenated HbS aggregates into a polymer because the deoxygenated confirmation allows a sufficient number of inter-tetrameric contacts or bonds to supply enough energy to stabilize the polymer structure (Baldwin, 1975).

Anything that stabilizes the deoxy-state relative to the oxy-state, such as the formation of a HbS polymer, will decrease the overall oxygen affinity of haemoglobin (Seakins et al., 1973). This hypothesis is supported by measurements of the oxygen affinity of concentrated HbS. Whereas in dilute solutions the oxygen affinities of Hb A and S are identical, in concentrated solutions or cells the oxygen affinity of HbS is markedly lowered (Winslow, 1978). The effects of pH and 2,3-diphosphoglycerate (2,3-DPG) on gelation are also mediated primarily through this mechanism. That is, lowering the pH from 8.5 to 6.5 decreases oxygen affinity via the Bohr effect, which increases the amount of deoxy HbS at any given oxygen tension value and therefore promotes gelation.
Similarly, increasing concentrations of 2,3-DPG stabilize the deoxygenated structure and enhance gelation (Kilmartin and Rossi-Bernardi, 1973). In addition however, other workers suggest that even in the absence of oxygen, a small decrease in pH may slightly enhance polymerization (Briehl and Evert, 1973).

Gelation is very dependant on deoxy HbS concentration (Singer and Singer, 1953). At a fixed temperature, if the concentration of deoxy HbS is raised it will eventually reach an equilibrium solubility beyond which the haemoglobin will come out of solution and form a gel.

At the high haemoglobin concentrations at which gelation occurs, this volume occupied by the individual molecules increases the extent and rate of reaction. This process is due to an increase in effective concentration of haemoglobin because of the decrease of surrounding solvent molecules (Ross et al., 1977).

Under usual experimental conditions (0.15M potassium phosphate, pH 7.15 at 20°C) the solubility of deoxy HbS is 20.8g per decilitre (Hofrichter et al., 1976). Since the red cell’s mean corpuscular haemoglobin concentration is usually greater than 30g per decilitre, upon deoxygenation the intracellular HbS will gel. In fact, gelation occurs with only partial deoxygenation.
2.4. RED BLOOD CELLS AND SICKLE RED CELLS.

Contained within the normal erythrocyte is a 5mM solution of haemoglobin, constituting 97.5 per cent of the cytoplasmic protein by weight (Harris and Kellermeyer, 1970). The other 2.5 per cent is primarily made up of protein systems that support and regulate the function of haemoglobin as well as maintaining the integrity of the cell. These proteins provide energy to maintain the ionic composition and water content of the cell; as well as supplying a reducing potential to keep haemoglobin in the ferrous form.

Surrounding the concentrated solution of haemoglobin is the red cell membrane, which constitutes a small percentage of the mass of the cell (Harris and Kellermeyer, 1970). It is composed of a bilayer of lipids and sterols in which a mass of proteins are distributed. Some of these proteins are involved in active ion transport, which keeps the intracellular Na+ and Ca2+ concentrations low and that of K+ high as compared to plasma levels. Other proteins, for example, actin and spectrin are found along the cytoplasmic membrane surface and are thought to form a filamentous cytoskeleton that anchors other membrane proteins and provides flexibility to the erythrocyte.
Flexibility is crucial for a red cell measuring 8 micrometres in its greatest diameter to traverse the 3 micrometres or smaller capillaries and pass through the interendothelial slits of the splenic sinuses (Weiss and Tavassoli, 1970). As erythrocytes age, this flexibility appears to decrease. This effect may be a factor in the eventual removal of erythrocytes from the circulation, although the mechanism by which this natural process occurs is not fully understood.

Gelation of sickle haemoglobin in the intact red cell can be observed in vitro by a variety of physical techniques: optical birefringence (Benesch et al., 1977), x-ray diffraction (Magdoff-Fairchild, 1972) and electron microscopy (Stetson, 1966). Intra-cellular polymerization in vitro is accompanied by deformation of the red cell (commonly termed as sickling) into characteristic sickle and holly-leaf forms.

For many years it has been known that the blood of patients with sickle cell anaemia contains erythrocytes that are irreversibly sickled (Diggs and Bibb, 1939). The proportion of these irreversibly sickled cells (ISC's) in the circulation varies from 5 to 50 per cent but fluctuates within narrow limits for each patient (Bertles and Milner, 1968). These cells are characterized by their inability to resume a normal biconcave disc shape even after reoxygenation (Bertles and Döbler, 1969).
Even though sickle red-cell membranes initially appear normal, repeated cycles of sickling and unsickling in vitro lead to permanent abnormalities that cause the formation of ISC's (Padilla et al., 1973). Precursors of ISC's appear to be reversibly sickled cells, but the mechanism of the transformation to ISC's is unclear. Because the ISC's may be relatively young, it has been postulated that a certain population of sickle cells may be unprotected from sickling, and therefore, be predisposed to irreversible sickling (Bertles and Milner, 1968).

Changes similar to, but more marked than those seen in reversibly sickled cells have been noted in ISC's. They have increased haemoglobin concentrations, decreased ATP levels, increased intracellular calcium and increased haemolysis coupled with loss of membrane deformability (Serjeant et al., 1969). Not only do these cells maintain their abnormal shape upon reoxygenation (Bertles and Dobler, 1969), but this abnormal shape persists even after the haemoglobin has been removed, and this phenomenon has been attributed to as yet incompletely understood permanent changes of membrane proteins (Lux et al., 1976).
2.5. THERAPEUTIC APPROACHES.

2.51. GENETIC COUNSELLING.

The gene for HbS is inherited in a Mendelian fashion. Therefore, it would seem possible to reduce the number of children born with the disease by large scale screening and genetic counselling of populations with a high frequency of the sickle cell gene. Large-scale screening programmes have not been popular and have been criticized because it was thought that it might lead to the detection of illegitamacy (Acquaye et al., 1981).

In Ghana, genetic counselling has meant informing the population about the social and clinical implications of the disease and educated couples about the probability of disease in their offspring (Konotey-Ahulu, 1975).

Another proposed method for the prevention of sickle cell disease is to terminate pregnancies after in utero diagnosis of a foetus with sickle cell disease. A few complications resulting in the loss of foetuses have however been reported (Kan, 1977). The disease is found predominantly in Black populations and genetic counselling poses an ethical problem; different communities will react differently to these approaches.
For example, in African states abortion is illegal and the disease is not as severe as that in patients from the U.S.A. because patients from Africa tend to have a higher level of foetal haemoglobins in their erythrocytes (Konotey-Ahulu, 1977).

2.52. CONSERVATIVE TREATMENT.

The conservative method of treatment is based on the fact that when sickling occurs in vivo it is usually found to be associated with a qualitative or quantitative change in the body. Changes which cause sickling in vivo include fever, dehydration, acidosis, tonsilitis, pneumonia, leg ulcers and hypoxia. Such factors are identified, anticipated, prevented or reversed (Acquaye et al., 1981).

After sickling has occurred, even though the patient may be in severe pain, the impulse to give potent analgesic immediately is resisted until the cause of the crisis is identified and a specific treatment begun. Otherwise, the analgesic will have to be repeated because they do not clear the crisis and as soon as the drug is metabolized or excreted the pain comes back. Repeated administration of analgesics are not desirable owing to toxic side-effects and possible addiction.
Intravenous fluids are given liberally to correct changes in the internal environment. Blood is not given routinely, since repeated transfusions have been associated with hypertension, convulsions and cerebral haemorrhage (Royal and Seeler, 1978). Even though the results of treatment are not consistent, conservative treatment is the preferred method for the management of the sickle cell disease; therefore the search for a suitable therapeutic agent is still of great priority.

2.53. CHEMOTHERAPY.

When HbS molecules are provoked to undergo gelation there is a pronounced delay before the appearance of deoxy HbS polymers (Hofrichter et al., 1974). Therefore an increase in the delay time; permitting erythrocytes to escape the narrow capillaries before gelation begins, would reduce the effects of the disease. Strategies for increasing the delay time include the direct inhibition of polymerization with hydrophobic molecules that competitively bind, non-covalently to the sites of inter-molecular contact; inhibition of polymerization by covalently modifying HbS to increase oxygen affinity and by decreasing intracellular haemoglobin concentration.
An ideal therapeutic agent for the possible treatment of sickle cell disease should have the following characteristics: the agent must a) pass through the erythrocyte membrane without altering membrane structure and function; b) inhibit polymerization of deoxy HbS molecules without altering the oxygen binding properties; c) be non-toxic in all tissues, and d) restore to normal the oxygen-carrying characteristics of HbS within the cell.

A. NON-COVALENTLY BOUND INHIBITORS.
Murayama (1966), showed that gelation of deoxy HbS decreased with increasing temperature. He proposed that the conformational changes observed in deoxy HbS were due to the HbS molecule forming hydrophobic bonds between interacting haemoglobin tetramers. It was subsequently suggested that molecules such as urea and the relatively short peptides from the amino acid sequence corresponding to the contact regions of the deoxy HbS polymer would be the most effective inhibitors of HbS gelation (Nalbandian et al., 1971).
In 1972, Nalbandian demonstrated that 1.0M urea in invert sugar reversed sickling in vivo, and 0.0062M could produce a complete inhibition of sickling. The results of clinical trials in the U.S.A. and tropical Africa were disappointing. Moreover, side effects such as dehydration, headache and vein irritation were reported. Substituted urea's seen to act by a similar mechanism (Elbaum et al., 1974), however more laboratory tests need to be done before testing on patients.

When administered, urea is excreted unchanged. Methylurea seems to be decomposed in vivo, while arylurea's, e.g. phenylurea is hydroxylated to p-hydroxyphenylurea which is excreted conjugated with glucuronic acid.

L-phenylalanine (Noguchi and Schechter, 1978); benzyl alcohol alcohol (Ross and Subramanian, 1977); and some short hydrophobic peptides (Votano et al., 1977) have been found to prevent gelation of deoxy HbS by hydrophobic interactions. The problem with these compounds is that they are not transported readily across the erythrocyte membrane in amounts required to prevent gelation of deoxy HbS. Therefore, they do not prevent sickling when incubated with sickle erythrocytes.
In recent years these compounds have been modified structurally and results have been encouraging. Thus L-phenylalanine benzylester, an analogue of phenylalanine, has been found to have anti-sickling properties. The mechanism of action of this agent as an anti-sickling agent is not yet completely understood, but it is thought to act by non-covalent interaction of the products of hydrolysis with HbS and by interaction with the erythrocyte membrane (Gorecki et al., 1980). Benzyl alcohol and L-phenylalanine are both oxidized in vivo to benzoic acid, which is then conjugated with glycine and excreted as hippuric acid.

Very recently, novel agents have been developed as potential therapeutic agents in the treatment of sickle cell disease. Asakura et al. (1981), reported that Piracetam, a 2-oxo-1-pyrrolidine acetamide, prolongs the delay time prior to gelation significantly as well as increasing the elasticity of the red cell membrane. The clinical use of this drug could be limited since relatively high concentrations of the drug are required to reveal anti-sickling effects.
B. COVALENTLY BOUND INHIBITORS.

In 1971, Cerami and Manning suggested that since urea is rapidly cleared from the blood, it seemed likely that the reported long-term clinical improvement of the sickle cell patient would be due to cyanate, which is usually in equilibrium with urea in solution. Therefore, they investigated the effects of potassium cyanate on the sickling of erythrocytes and found that the cyanate permanently prevented sickling in vitro and in vivo by reacting specifically with the amino-terminal groups of both alpha- and beta-globin chains of haemoglobin. Unfortunately, the specificity is not absolute in vivo. Cyanate carbamylates also free amino groups in lens and myelin of peripheral nerves resulting in the development of peripheral neuropathy and cataracts (Tellez-Nagel et al., 1977).

Subsequent clinical trials in man revealed adverse effects such as loss of weight and appetite, tingling burning sensations in the extremities of the body and nerve disorders (Peterson et al., 1974). In an attempt to reduce the side effects of cyanates, extra-corporeal treatment was tried. In this procedure some of the circulating erythrocytes were removed from the body and treated with cyanate.
The excess cyanate was washed away and the erythrocytes reinfused into the blood. However, the results of subsequent management of the disease were not encouraging.

Other anti-sickling agents that act by binding covalently to HbS are been developed. A common property of these compounds is that they increase the oxygen affinity of the haemoglobin molecule, therefore, they might cause functional anaemia (Dean and Schechter, 1978).

C. COMPOUNDS CONSIDERED TO AFFECT MEMBRANES.

Robinson (1979), demonstrated that steroid hormones inhibited the development of sickling in sickle cell disease and suggested that the hormones acted by stabilizing the erythrocyte membranes. Subsequent clinical trials yielded contradictory conclusions. The negative observations may be attributed to differences in dosage. Prasad et al. (1976), have shown that zinc has a potential for the treatment of sickle cell disease. Zinc treatment reduces the number of ISC's in the patient and might therefore, slow organ damage. Exactly how zinc prevents sickling is not completely understood, but it may act by inhibiting calcium-ATPase by displacing calcium (which increases the tendency of erythrocytes to sickle) from the erythrocyte membrane. Clinical trials are still in progress.
Because gelation of deoxy HbS is markedly dependent on the concentration of haemoglobin, any factor that would result in an effective decrease in the intracellular concentration of HbS would minimize sickling.

In 1978, Dean and Schechter suggested that pharmacological agents that alter membrane permeability and allow an influx of water, could be beneficial in controlling sickling. Since then, a number of compounds, including phenylalanine benzylester (Peterson et al., 1974) have been shown to prevent sickling, probably also by causing sickle erythrocytes to swell. This approach seems to be promising, however, a compound that effects both the membrane permeability to produce a limited swelling of the cell and the haemoglobin molecule by increasing its solubility or increasing the lag time for polymerization of deoxy HbS, would be a better anti-sickling agent than one that effects the haemoglobin molecule or membrane alone.
In 1983, Franklin et al. showed that a dipeptide L-lysyl-L-phenylalanine (Lys-Phe) inhibited both cell sickling and the gelation of solutions of HbS in vitro (Fig. 2.). This result, together with measurements of uptake both into the cell and onto the cell membrane showed that the compound produces a membrane-mediated anti-sickling effect in addition to an effect on haemoglobin in solution within the cell. The membrane effect was not due to a change in cell volume. Under normal incubation conditions the dipeptide did not readily cross the red cell membrane (Franklin et al., 1983). Therefore, encapsulation involving the entry of Lys-Phe into the cytosol of the erythrocyte would be an interesting in vitro model to study Lys-Phe-HbS interactions.
FIG. 2. THE STRUCTURE OF L-LYSYL-L-PHENYLALANINE.

L-LYSYL-L-PHENYLALANINE

\[
\begin{align*}
\text{NH}_2 & - \text{CH} - \text{CONH} - \text{CH} - \text{CO}_2\text{H} \\
& | \quad | \quad \quad (\text{CH}_2)_4 \text{NH}_2 \quad \text{CH}_2 \text{Ph}
\end{align*}
\]

329.9 Daltons
D. GENETIC ENGINEERING.

The use of genetic engineering can be envisaged to cure inborn errors of metabolism, including sickle cell disease. The normal genes for haemoglobin could be manufactured by recombinant D.N.A. techniques and mixed with bone marrow cells removed from the patient. Then the bone marrow cells, bearing the healthy genes could be injected back into the patient to direct the production of normal haemoglobin.
3. **GASTRIC ACID SECRETION.**

3.1. **INTRODUCTION.**

Gastric secretion of hydrochloric acid depends on the mass and functional capacity of respective secretory elements in the gastric mucosa and the action of various hormonal and nervous stimulants and inhibitors. The rate of secretion and the composition of gastric juice show profound alterations in response to physiological stimuli such as a meal, which may affect the secretory activity of gastric glands either directly by luminal contents or indirectly through gastrointestinal hormones and other chemical messengers delivered to the secretory cells by endocrine, paracrine or neurocrine pathways. Increased knowledge of gastric physiology has clearly shown that a number of hormonal peptides present both in the endocrine-paracrine cells and in the nerves of the digestive system are involved in the control of acid secretion.
3.2. SECRETION OF HYDROCHLORIC ACID.

Hydrochloric acid is secreted into the gastric lumen by the oxyntic cells. The primary secretion of these cells, as it appears in the intracellular canaliculi, has a very high concentration of acid, reaching approximately 170mM which is more than three million times greater than in blood or tissues. The secretory process requires two major intracellular steps: production and active transport of hydrogen ions, both of which consume energy and oxygen. The energy is generated in the oxyntic cells mainly by aerobic metabolism and involves the production of high energy phosphate bonds. Oxygen consumption is tightly linked to the secretion of acid and more than one hydrogen ion is secreted per oxygen molecule (Hersey, 1974).

There are conflicting studies on the source of substrate pool, but probably both, fatty acids and glucose are metabolized to yield the products involved in acid secretion, namely the hydrogen ion itself, ATP, carbon-dioxide and the hydroxyl radical (Hersey, 1977; Sachs et al., 1977). The source of the hydrogen ions for the secretion of acid and the means of the delivery of metabolic energy to the hydrogen-ion pump are not known but two major theories have been proposed in this regard.
According to the redox theory, hydrogen ions are directly generated from the oxidation-reduction process and delivered to the secretory surface area via the electron transport system. The ATP theory suggests that ATP generated from the substrate metabolism provides the necessary energy via a specialized ATPase. Gastric mucosa contains several ATPase's but a specialized K+-stimulated ATPase appears to represent an integral part of the acid secretory mechanism. This ATPase was recently found in the vesicles isolated from the oxyntic cells and shown to be capable of concentrating acid in the presence of exogenous ATP (Forte and Lee, 1977).

The concentration of hydrogen ions in the gastric juice is a function of the rate of secretion. As the rate of secretion increases, the hydrogen ion concentration rises and the sodium concentration falls, whereas the potassium and chloride concentrations remain within a fairly narrow range (Hollander, 1952).
3.3. GASTROINTESTINAL HORMONES AND THE INHIBITORY MECHANISMS OF GASTIC ACID SECRION.

INTRODUCTION.

The observation that secretion of gastric acid is markedly increased after the ingestion of food and virtually abolished during the interdigestive period, formed a basis for the concept that gastric secretion depends on the interplay of stimulatory and inhibitory influences arising from the stomach and the intestines. Through interactions between nervous and hormonal stimuli, gastric secretion is controlled by complex feedback mechanisms (Konturek, 1974). The known initiators of inhibition are acid in the stomach and duodenum, fat or hypertonic solutions in the duodenum and hyperglycaemia.

A. ANTRAL AND FUNDUS FEEDBACK INHIBITION OF GASTRIC SECRION. ROLE OF GASTRONE AND SOMATOOSTATIN.

Woodward et al. (1954) found that bathing the antral mucosa with acid, suppressed gastrin release and gastric acid secretion. Lowering gastric pH 5.5 to 1.0 completely suppressed gastrin release in response to an amino acid or peptone meal and resulted in a marked inhibition of acid secretion.
This inhibition probably occurs under physiological conditions, as after a mixed meal, intragastric content reached a pH level of about 2-3; that was enough to suppress gastrin release and to inhibit gastric acid secretion (Konturek, 1980). Suppression of gastrin release is the only recognized mechanism by which acid bathing the pyloric gland inhibits acid secretion (Konturek, 1974).

1. GASTRONE.

A possible candidate for the active principle released by antral acidification is gastrone (Grossman et al., 1974), an inhibitor substance present in the achlohydric gastric juice of pernicious anaemia patients. Gastrone has been identified by immunocytochemistry in antral mucosa and found to be an inhibitor of acid secretion, but it has not been isolated and chemically characterized. Its relationship to other inhibitory substances found in saliva (sialogastrone) and in the urine (urogastrone) remains to be established, and the final evidence that it is involved in the physiological mechanisms controlling gastric secretion is still to be obtained.
2. SOMATOSTATIN.

Somatostatin is a potent inhibitor of gastrin release and of gastric acid secretion induced by a meal and exogenous stimulants acting directly on the oxyntic cells (Schusdziarra et al., 1978). It was also reported that immunoreactive somatostatin is released under basal conditions into the blood draining the stomach and the pancreas and that gastric or duodenal acidification results in a marked rise in the plasma level of somatostatin. This suggests that somatostatin may be involved in the antral and duodenal inhibitory mechanisms. It has also been postulated that somatostatin may be a mediator of the inhibitory mechanism of acid secretion arising from the oxyntic mucosa (Schusdziarra et al., 1978).

B. DUODENAL FEEDBACK INHIBITION OF GASTRIC ACID SECRETION.

ROLE OF SECRETIN.

Instillation of large amounts of acid into the duodenum inhibits both gastrin and histamine-stimulated gastric acid secretion in the dog (Konturek et al., 1971) and man (Konturek, 1970). The physiological significance of acid in the human duodenum in the inhibition of gastric secretion has not yet been established. It has been reported that duodenal acidification in man inhibits gastrin-induced gastric acid secretion, only when acidification is severe and prolonged (Wormsley, 1970).
1. SECRETIN.

The principle human mechanism of gastric inhibition by duodenal acidification is the release of secretin from the endocrine S cells present in the duodenal mucosa (Konturek, 1980). Although exogenous secretin does inhibit gastrin, histamine and food-induced acid secretion, it seems unlikely that it is the only mediator of gastric inhibition by duodenal acidification (Johnson and Grossman, 1972). Whatever the significance of secretin in gastric acid inhibition in man, there does not appear to be any difference between the normal and duodenal ulcer patients in the release of endogenous secretin in response to exogenous hormone (Dalton et al., 1976).

C. INHIBITION OF GASTRIC ACID SECRETION BY FAT AND HYPERTONIC SOLUTIONS IN THE DUODENUM.

ROLE OF CCK, GIP AND VIP.

Fat introduced into the duodenum in an absolute form inhibits gastric secretion. This inhibition is effective against gastrin-, histamine- and food-induced acid secretion (Johnson and Grossman, 1972), and can be demonstrated in both healthy subjects and in duodenal ulcer patients (Gross et al., 1978).
The mechanism by which fat inhibits acid secretion is not fully understood, but gut hormones are involved (Rayford et al., 1978). A neural inhibitory reflex has been proposed as the mode of action of fat in the duodenum, since the inhibition was reported less pronounced after vagotomy. However, the vagus may interact with inhibitory hormones at the level of the oxyntic cells or may facilitate the release of these hormones from the gastrointestinal tract (Konturek, 1980).

1. **CHOLECYSTOKININ (CCK).**

A number of studies have been undertaken to identify the hormones that are released by fat and involved in gastric acid inhibition. It is now well established that fat releases CCK from the intestinal mucosa but there is little doubt that this hormone cannot be solely responsible for inhibition, since fat induces suppression of histamine-stimulated acid secretion that cannot be reproduced by CCK or secretion regardless of the dose used (Johnson and Grossman, 1969).
2. **GASTRIC INHIBITORY PEPTIDE (GIP)**.

Fat also releases GIP from the intestinal endocrine-type K cells (Brown et al., 1978). GIP is a 43-amino acid peptide that inhibits gastric acid secretion in the dog during stimulation by pentagastrin, histamine, insulin or food. Large doses of GIP also inhibited gastrin release in dogs.

Recently, GIP was also demonstrated to inhibit pentagastrin or meal-induced gastric secretion in man, but doses used, increased serum GIP levels two-to-five fold higher than those measured after ingestion of a mixed meal (Arnold et al., 1978). Further studies are still needed to determine how much of the inhibition of acid secretion by fat can be accounted for by release of GIP.

3. **VASOACTIVE INTESTINAL PEPTIDE (VIP)**.

VIP is another candidate for the agent that is released by fat and inhibits gastric secretion. VIP contains 28 amino acid residues and is present both in the gastrointestinal mucosa and also in nerve fibres of the myenteric plexus. It is a potent inhibitor of pentagastrin and histamine-stimulated gastric secretion (Konturek et al., 1975). It suppresses food-stimulated gastric secretion and gastrin release.
4. HYPERTONIC SOLUTIONS.

Hypertonic solutions instilled into the duodenum inhibit gastric secretion (Konturek and Grossman, 1965), but neither the mechanism of this effect nor the extent to which it operates under physiologic conditions is known. Since the inhibition persisted after the denervation and transplantation of the fundic pouches in dogs, it was concluded that it is hormonal in nature. In the case of hypertonic glucose in the duodenum, at least two mechanisms are involved; one is hyperglycaemia, which inhibits gastric secretion by central suppression of vagal activity, and the other is GIP, which is released by glucose in the gut and affects gastric secretion (MacGregor et al., 1976).
3.4 ISOLATION AND STRUCTURE OF UROGASTRONE.

3.4.1 INTRODUCTION.

The clinical observation of the very low incidence of peptic ulceration during pregnancy led to a finding that extracts of the urine of pregnant women had a beneficial effect on experimental ulcers in dogs (Gray et al., 1939). It was later reported that not only pregnancy urine but also that from all females and males, contained a potent inhibitor of gastric acid secretion (Friedman et al., 1939). Sandweiss and Friedman (1942), named the anti-ulcer factor, antelone, and regarded it as having a therapeutic effect on ulcers without depressing gastric acid secretion. The anti-secretory agent was thought to be a separate entity and was called urogastrone because its actions resembled those of the postulated duodenal hormone enterogastrone (Gray et al., 1940).

To establish the true nature of these agents, however, and in particular their possible role in the therapeutic control of peptic ulceration it was necessary to make a full chemical identification. Many attempts have been made to isolate urogastrone, and probably the most highly purified sample on record was that obtained by Gregory (1955). This was described as a combination of a golden yellow fluorescent pigment and a protein of relatively low molecular weight.
Others have produced additional evidence of a protein structure (Rosenoer and Schild, 1962), but some recent work has led to the suggestion that urogastrone is a high molecular weight glycoprotein (Morimoto and Yamamoto, 1969). Nevertheless, its exact nature, source and physiological role has remained unknown.

Highly purified samples of urogastrone from normal urine have now been obtained by means of a twelve-stage purification process involving ion exchange, partition and gel chromatography (Gregory, 1975). There seems to be more than one inhibitor of gastric acid secretion in urine but the main active components were eventually found in yields of less than 1 mg per 1000 l of urine. Two closely related products were isolated and for these the original name of urogastrone has been used. The two urogastrones were shown to be water-soluble polypeptides of relatively low molecular weight and the difference between them could be shown by various physical techniques, including acrylamide gel electrophoresis. They caused an intense inhibition of gastric acid secretion in cats and dogs and seemed to be biologically indistinguishable. Because of the small amounts of material available, the two peptides were sometimes used together. Doses as low as 0.25 micro-grams per kg produced 60-80% inhibition in Heidenhain pouch dogs stimulated to near maximal levels of acid secretion with pentagastrin or histamine. (Gregory, 1975).
Work with less pure material had established that at doses producing strong inhibition of acid secretion, urogastrone did not affect other secretions such as pancreatic, biliary or salivary secretion and the effects seemed to be quite specific for the stomach (Gerring et al., 1974). It did not affect blood pressure, pulse rate or body temperature. Also, it did not seem to be inhibiting acid secretion by causing a restriction of mucosal blood flow.

3.42. ISOLATION.

The earliest methods for processing urine were based on benzoic acid precipitation; some use was also made of ethanol and ammonium sulphate precipitation and charcoal, which proved to be an effective adsorbent (Gregory, 1955). For routine large-scale use, however, precipitation of the active material with tannic acid was found to be convenient. Volumes of approximately 400 litres daytime male urine were processed daily to give a solid, 20 to 30 mg/litre urine, which retained potency indefinitely when stored at -40°C. Subsequent studies showed that approximately 30 micro-gram/litre urine were obtained when measured by radioimmunoassay (Gregory, 1980).
Inhibitory effects of urine extracts have been described in a number of species, and dogs with denervated fundic pouches, with histamine or pentagastrin as stimulant, were used to quantitate the gastric secretory response to purified fractions. For routine, rapid testing of large numbers of fractions, rats with a simple stomach perfusion gave quick positive or negative evaluations.

The two isolated polypeptides, designated beta- and gamma-urolagastone were found to consist of 53 and 52 amino acid residues respectively. The full structures of these peptides were established and it was found that the difference resided only in the C-terminal arginine residue which was absent in the gamma peptide (Gregory et al., 1975). The two urolagastones were each obtained in yields of less than 1 mg/1000 litres urine; they were both acidic polypeptides and readily water-soluble. Biological activity was retained after the peptides were kept in solutions of pH 1 to 11 at 37°C for extended periods and also in solutions of strong organic acids such as trifluoroacetic acid. They were found to be resistant to degradation by mammalian proteolytic enzymes; treatment with trypsin or pepsin at 37°C for 12 hours left the molecule intact (Gregory et al., 1972).
3.43. STRUCTURE.

It was necessary to break the disulphide bonds in the urogastrones to enable clean enzymic digests to be prepared. Both S-carboxymethyl and S-carboxamidomethyl derivatives were made, and these had the same molecular size and amino acid composition as the parent molecule, thus indicating that they were single polypeptide chains (Gregory and Preston, 1977).

They were susceptible to a number of proteolytic enzymes which were useful giving smaller peptides fragments that were purified and their sequences established using the dansyl-Edman technique. An enzyme isolated from the fungus Armillaria mellea had good specificity for bonds on the amino side of lysine, and this gave a good limited breakdown of the urogastrone derivatives. Partial acid hydrolysis gave additional peptide fragments, and with many small peptides from the different degradations, the overlapping sequences established the structure of beta-urogastrone. As work progressed it became apparent that gamma-urogastrone had the same sequence apart from being one amino acid shorter (Gregory, 1975). The directions of the disulphide bonds were indicated from partial acid hydrolysis of the intact molecule. The structure of beta-urogastrone was found to be a single polypeptide chain of 53 residues with three internal disulphide bonds (Fig 3), (Gregory and Preston, 1977).
FIG. 3. AMINO ACID SEQUENCE OF BETA-UGASTRONE.

RESIDUES ALTERED IN MOUSE EPIDERMAL GROWTH FACTOR ARE GIVEN IN PARENTHESIS.
[Tyr, Pro, Gly]  [Ser]  [Tyr]
H, Asn, Ser, Glu, Cys, Pro, Leu, Ser, His, Asp, Gly.

[His]  [Gly, Asn]
Tyr, Met, Cys, Val, Gly, Asp, His, Leu, Cys, Tyr.

[Ser]  [Ser]  [Thr]
Ile, Glu, Ala, Leu, Asp, Lys, Tyr, Ala, Cys.

[Ile]
Tyr, Gly, Val, Val, Cys, Asn.

[Ser]  [Asp]  [Thr]
Ile, Gly, Glu, Arg, Cys, Gln, Tyr.

[Arg]
OH, Arg, Leu, Glu, Trp, Lys, Leu, Asp, Arg.

6201 Daltons
Isoelectric point = pH 4.5
3.44. ACTION ON GASTRIC SECRETIONS.

Urogastrone strongly suppresses acid secretion induced by the action of histamine and also the hormonal type of response to pentagastrin, the cholinergic stimulation of methacholine or the physiological stimulus of a test meal in dogs (Gerring et al., 1974). It is also effective in cats, monkeys and rats, although the latter species is somewhat less sensitive. The dominant effect is on the volume of secretion. Acid concentration does fall slightly, pepsin concentration increases slightly and rises further as the levels of secretion rise toward the initial levels.

Doses as low as 0.25 micro-gram/kg/hr intravenously gave inhibition of submaximal pentagastrin responses in man to the extent of 80%. Secretion stimulated by histamine or insulin was similarly suppressed without clinical side effects. Although acid and intrinsic factor outputs were reduced, pepsin output was less obviously affected but rose after completing the infusion of inhibitor (Gillespie et al., 1974). In patients with Zollinger-Ellison syndrome the basal gastric secretion was reduced by 50 to 80% using the standard infusion of 0.25 micro-gram/kg/hr. (Elder et al., 1974).
Studies in ulcer patients confirmed the ability of urogastrone to suppress basal acid secretion; maximal secretion in response to pentagastrin was blocked to the extent of 50% by the standard infusion rate, and it remained equally effective after five daily infusions to these patients (Koffman et al., 1977).

3.45. RELATIONSHIP TO MOUSE EPIDERMAL GROWTH FACTOR.
When urogastrone was characterized it was clearly different from known gastrointestinal peptides, however, a striking correlation with mouse epidermal growth factor (mEGF) emerged subsequently. This peptide, which was present in male mouse submaxillary glands at over 0.1% of the wet weight, was also a polypeptide of 53 amino residues (Savage et al., 1972) with only 16 of these changed relative to urogastrone (Gregory, 1975). The substitutions occur fairly evenly across the whole molecule, and 14 of them were compatible with single base changes in the triplet codons of the genetic code. Although urogastrone is rather more acidic than mEGF, predictably the changes would have little effect on the overall conformation of the two molecules (Gregory and Preston, 1977).
Early work with mEGF showed that it possessed the remarkable property of causing premature eye opening when injected daily into newborn mice (Cohen, 1962), and it has subsequently been shown to stimulate the growth of epithelial cell tissue in a variety of preparations (Cohen and Savage, 1974). The behaviour of mEGF on thin-layer acrylamide gel electrophoresis was compared with the two urogastrones and it was found that mEGF moved more closely to the anode at pH 8.9 than the two urogastrones, which differ by the single arginine residue (Gregory, 1975).

The potency of this preparation was confirmed by its action on newborn mice. mEGF given as a subcutaneous injection of 5 micro-grams in 25 micro-litre saline caused the eyes to open on day 10 compared with day 13 in the controls (Gregory, 1975). It was found subsequently that this material would produce strong inhibition of gastric acid secretion in rats and dogs when given intravenously against exogenous stimuli (Gregory, 1975). The doses, however, of mEGF required to induce eye-opening ranged from over 300 micro-gram/kg/day initially to about 100 micro-gram/kg, whereas inhibition of acid secretion was obtained with less than 0.5 micro-gram/kg in dogs and less than 10 micro-gram/kg in rats.
The time course of inhibition was indistinguishable from that obtained by intravenous injections of urogastrone (Gerring et al., 1970). Maximum inhibition was obtained 15-30 mins after injection and secretion then gradually returned to the plateau levels after some 90 mins. Longer periods of inhibition could be obtained by giving the peptides subcutaneously at rather higher doses. It seemed, therefore, that mEGF had biological actions similar to those established for urogastrone and so urogastrone was examined to see whether it would produce the known effects of mEGF on newborn mice (Gregory, 1975).

At the established dose levels of 5 micro-gram/mouse/day it was found that urogastrone would also induce eye opening on day 10 compared with day 13 for the controls. Although the full spectrum of mEGF has not been studied there is substantial overlap in the properties of the two polypeptides.

As mentioned previously, the two peptides show remarkable structural similarities. Of the 53 amino acid molecules, 37 are common to both peptides. The 6 cysteines occupy the same relative positions and the 3 disulphide bonds are formed in the same direction. The 16 variable residues occur at intervals along the chains and of these, 14 are interpretable as single base changes in the coding triplets.
The 2 additional pairs are at position 28- Ser,Lys- and position 44-Thr,Tyr. The longest sequences common to both peptides consist of 5 residues but one of these, the C-terminal penta-peptide, does not seem to be necessary for inhibitory activity (Gregory, 1975).

The salivary glands of species other than the mouse are not a good source of the growth factor (Cohen, 1962), and only the male mouse provides substantial quantities. Females can be made to produce much larger amounts of EGF, however, as a result of androgen treatment. This sexual dimorphism does not normally apply to urogastrone and levels in urine are very similar for the human male and non-pregnant female (Fitzgerald et al., 1968).
3.46. ACTION ON CELLULAR PROCESSES.

The effects of urogastrone and mEGF were compared on cultured human fibroblasts (Hollenberg and Gregory, 1979). DNA synthesis and alpha-aminobutyric acid uptake were stimulated to the same extent (ED50 = 0.6 ng/ml), and at submaximal concentrations the effects of the two peptides were additive but gave the same maximal effect. Urogastrone or mEGF competed similarly with either of the peptides labelled with 125I, confirming that they shared a common site on the fibroblasts. Urogastrone and mEGF had similar behaviour in a number of systems that the much more readily available mouse peptide was often used routinely to study "urogastrone" actions in preliminary experiments.

Studies have been extended with urogastrone to a number of experimentally prepared ulcer systems. In reserpine-induced ulcers in guinea-pigs, infusions of urogastrone at 5 or 10 micro-gram/kg/hr markedly inhibited the formation of gastric erosions, and the acid and pepsin secretions were substantially reduced (Gregory et al., 1972). In rats, single acetic acid-induced duodenal ulcers showed a decrease of 50% compared with controls at a dose of 5 micro-gram/kg/hr s.c.; mEGF gave a similar result (Gregory et al., 1972).
Direct healing effects are not easily separated from the reduced acid secretion, but in rats, mEGF at 50 micro-gram/kg caused a 100% increase in ornithine and histidine decarboxylase activity in the stomach (Gregory, 1980). mEGF also stimulated ornithine decarboxylase activity in the stomach and duodenum of neonatal mice (Feldman et al., 1978). These two enzymes were associated with proliferating tissue and were also stimulated in mouse skin by the growth factor.

It is not known whether or not the immediate action on acid secretion and the more slowly observed mitogenic effects are manifestations of the same process. In the Zollinger-Ellison patients the decrease in acid secretion was accompanied by an increase in intrinsic factor output; thus all parietal cell processes were not inhibited. All stimulants of acid secretion are blocked by urogastrone, and this could possibly be because the cell is switched to an alternative process.
3.5. **AIMS OF RESEARCH.**

The guidelines to this project were originally specified by G.D. Searle and Co.Ltd.

Initially, the two peptides were encapsulated into rat erythrocytes using the minimum damage system, in an attempt to produce viable loaded cells. The survival of carrier erythrocytes was monitored by labelling the cells with fluorescein isothiocyanate. After encapsulation, the peptides were assayed by radio-active methods to determine the percentage encapsulation.

The leakage of Lys-Phe and urogastrone from erythrocytes in vitro was measured by the rate of release through dialysis sacs, and the integrity of the peptide determined by thin-layer chromatography.

Pharmacological or biological activity of encapsulated preparations was then tested. In the case of Lys-Phe, an attempt was made to encapsulate the dipeptide into intact ss-RBC in situ. Encapsulated urogastrone was tested for biological activity using the perfused rat stomach or the pylorus-ligated rat preparations.

In an attempt to improve the storage times for these preparations, erythrocytes were stored for extended periods on either a gelatin or agarose gel with the inclusion of various nucleotides.
4.1. **ANIMALS.**

In vivo experiments were performed using male Wistar strain rats (Bantin and Kingman, Hull). The animals were young adults of 200-300g body weight (unless otherwise specified), and were allowed a diet of food and water ad libitum, again unless otherwise stated.

4.2. **ANAESTHESIA.**

For all the routine encapsulation experiments, the rats were anaesthetised using an inhaled gaseous mixture of 3% halothane in oxygen (300 cubic cm/min), and nitrous oxide (1000 cubic cm/min), using a Boyle's veterinary anaesthetic apparatus (British Oxygen Co.). Total anaesthesia was maintained for the appropriate length of time using a mixture of 1.5% halothane in oxygen and nitrous oxide.
4.3. Modified K+-Reversed Hanks Physiological Medium.

The medium used for swelling and resealing erythrocytes during the encapsulation procedure (and also for other experimental work), was based on that originally specified by Hanks (1948), for tissue culture.

The K+-reversed version of such media means that the sodium and potassium ion concentrations are reversed so that they more closely match the ion concentrations found in the interior of the cells. This medium has been used for a number of years in the Aston and Oxford laboratories for encapsulation work and a number of modifications have been made to it (Pitt et al., 1983a). It was originally specified that the indicator phenol red was present to warn of pH changes. Subsequently, it was found that its presence was not relevant to the encapsulation work, and therefore it was omitted. Problems have been encountered with precipitation in the media and these were thought to be due to the relative insolubility of calcium and magnesium carbonates. After work by Pitt et al. (1983a), it was decided to omit calcium chloride and magnesium sulphate from the formula since no disadvantages were encountered in the use of the modified preparation.
Therefore, the composition of the modified K+-reversed Hanks medium used was:

KCL 10.18g/l
KH2PO4 0.1g/l
NaHCO3 1.273g/l
NaCl 0.316g/l
NaHPO4.2H2O 0.1g/l
GLUCOSE 2.0g/l
4.4. THE METHOD OF ENCAPSULATION OF AGENTS INTO ERYTHROCYTES.

4.4.1. INTRODUCTION.

During the procedure involving the encapsulation of agents in erythrocytes, care was taken throughout to avoid physical damage to the cells. Procedures such as mixing and transfers by pipette were done slowly and gently.

For in vivo experiments, all apparatus were sterilised by autoclaving. Water and saline solutions were also sterilised in the same manner. Hanks media was filtered via a Millipore (Millipore U.K., Harrow) filter. Since the drug solutions were of such small volume, they could not be filtered, however, they were prepared aseptically. For in vivo experiments, the encapsulation procedure was performed under aseptic conditions.
4.42. PROCEDURE.

1) Blood was removed from anaesthetised rats by cardiac puncture, and drawn into a heparinised syringe (0.5 ml containing 1000 units heparin/ml). For in vivo experiments approximately 4 ml of blood was taken since 1 ml of packed cells was to be used for the encapsulation procedure (1 ml packed cells is approximately approximately equal to 3.5 ml whole blood).

2) Erythrocytes were sedimented from whole blood by centrifugation at 600 g for 10 min. (Heraeus Christ Universal Junior 15 centrifuge). The plasma and the white cells (buffy coat) were discarded.

3) A 1 ml portion of the packed cells (1.04 ml on the pipette) was transferred to a clean test tube. The cells were then swollen by adding four volumes (i.e. 4 ml) of 0.66 isotonic modified reversed Hanks solution, and the contents were mixed gently by inverting the tube several times.

4) The suspension was centrifuged at 600 g for 5 mins and the supernatant was removed, leaving a noticeably larger volume of swollen cells.

5) A haemolysate was prepared separately by diluting 0.2 ml of the packed cells with an equal amount of distilled water (i.e. 0.2 ml). This was to provide a stock of cytosol constituents, thereby reducing the loss of these constituents when the cells were swollen.
6) A 0.2ml portion of the haemolysate was gently layered on top of the swollen cells, by running it slowly round the inside of the test tube, and at the same time, spinning the tube with the fingers. This was to provide a barrier between the cells and the aqueous material to be encapsulated so that the transfer could be accomplished with the minimum shock to the system.

7) An aqueous solution of the drug to be encapsulated was prepared and a 0.2ml portion was gently layered on top of the haemolysate layer. The test tube was gently inverted several times to mix the contents and then centrifuged at 500g for 2-3 mins to give a new haemolysate layer.

8) Drug addition was repeated as above until the swollen erythrocytes reached the point of haemolysis. This was the point when there was a sudden increase in the transparency of the suspension, darkening of the supernatant layer, the appearance of a few ghost cells and a poorer separation on centrifuging. The amount of drug which could be added depended on the nature of the drug itself i.e. molecular weight and concentration.

9) When the point of haemolysis was reached, the cells were resealed by restoration of their normal tonicity. This was achieved by the rapid addition and immediate shaking of the calculated amount of modified reversed Hanks medium at 10 times the eutonic strength.
The volume of 10 times Hanks medium added was calculated according to the following formula:

\[ \text{vol of water added} = \text{vol of cells (e.g. 1)} \times 0.9 \times 0.7 \]

(N.B. 0.9 = Salinity of cells
0.7 = Salinity of Hanks) = 1.29

Therefore, 0.29 ml of water was added.

Now total water = 0.29 + 0.1 (half of haemolysate) + 0.2 (if 0.2 ml water added)

= 0.59 ml

Therefore, volume of 10X Hanks to be added = 0.59 = 0.065 ml.

10) The resealed cells were centrifuged at 500g for 5 mins and the supernatant discarded. The cells were then washed by the addition of 5 ml of eutonic modified reversed Hanks medium and again centrifuged at 500g for 5 mins. The supernatant was discarded and the washing procedure repeated to recover the packed erythrocytes encapsulating the drug.

The whole procedure of encapsulation lasted for approximately 2 hours. For intravenous administration, the packed cells were suspended in an equal amount of normal saline for injections (refer to section 4.44.)
4.43 F.I.T.C. LABELLING OF ERYTHROCYTES.

In order to monitor the presence and survival of drug-loaded or control cells during in vivo experiments, the cells were labelled with fluorescein isothiocyanate (FITC).

The packed cells were prepared as described in section 4.42. A saturated solution of FITC in isotonic modified reversed Hanks medium was prepared and filtered via a Millipore filter. 0.6 to 0.7 ml of this solution was added to each 1 ml of packed cells, and the suspension was left to stand for 1 hr. The cells were then washed with 7 ml of isotonic Hanks medium, three times.

The packed erythrocytes were then diluted with up to 0.5 ml normal saline and injected into the rat via the femoral vein (See section 4.44.).
4.44. INTRAVENOUS INJECTIONS OF ENCAPSULATED PREPARATIONS IN RATS.

For in vivo experiments, drug-loaded FITC labelled erythrocytes were injected back into the rats by a femoral vein injection according to the following procedure:

1) The animal was placed under light anaesthesia (3% halothane in oxygen and nitrous oxide). The thigh was then swabbed with Hibitane in 70% alcohol and shaved.

2) The femoral vein was exposed by making a slit above the vein; then the connective tissue around the vein was removed. The vein was made more prominent by pressing the leg at the back and also by warming the vein with the aid of an overhead lamp.

3) A 1ml syringe was filled with packed cells and the tip of the syringe was then filled with saline. The air bubbles were removed (hence saline), and the needle was inserted at a shallow angle into the vein. The contents of the syringe was injected slowly over about 30 secs.

4) A cotton wool swab was held over the vein during the needle withdrawal and held on with slight pressure for about a minute. The surrounding skin was cleaned with Hibitane solution and the wound was closed with 3-4 metal wound clips.
4.45. METHOD OF OBTAINING BLOOD SAMPLES AND ERYTHROCYTE COUNTS.

In order to determine the survival of FITC labelled erythrocytes in the rat, regular blood counts were obtained by the following method:

1) The animal was placed under light anaesthesia, and the very tip of the tail was cleaned and swabbed.

2) The extreme fleshy tip of the tail was then cut off. The tail was gently massaged to obtain a few drops of blood, and collected in a small tube, containing normal saline.

Since relative counts of labelled cells were required, it was not necessary to have absolute concentrations, so only approximate dilutions were required.

3) The cells were counted on an ultra-violet microscope (Zeiss, Universal) using a standard haemocytometer grid. Initially a count was made under normal (tungsten lamp) illumination to obtain the total count. An ultra-violet light (deuterium lamp) was then added and only the fluorescing cells were counted, i.e. the labelled cells. Therefore, the percentage of labelled cells could be calculated. At least a thousand cells were counted for each total count.

4) The first sample was taken about 5 mins after the injection (i.e. time=0), followed by another sample at 1 hr, 24 hr and daily thereafter.
4.5. THE ENCAPSULATION OF LYS-PHE INTO INTACT ERYTHROCYTES.

4.51. INTRODUCTION.

By the introduction of a radiolabel on one of the amino acids of the dipeptide, the amount of drug encapsulated in the erythrocytes could be determined. It was decided to label all the carbon atoms on the phenylalanine part of the dipeptide using either tritiated or carbon-14 as the radiolabel. Tritiated Lys-Phe was kindly supplied by G.D. Searle and Co. Ltd., High Wycombe, Bucks, whereas carbon-14 Lys-Phe was synthesized as described in section 4.52.

The loading of Lys-Phe in erythrocytes was assayed by liquid scintillation counting and the in vivo survival of loaded erythrocytes was monitored by labelling the cells with FITC.

The leakage of Lys-Phe from erythrocytes was investigated by conducting in vitro experiments using dialysis sacs, and also, experiments to verify the presence of the intact dipeptide were performed.

To test the encapsulated system, Lys-Phe was encapsulated into intact SS-erythrocytes and the cells were examined under the electron microscope for any evidence of an inhibition in sickling.

Lastly, this method of loading Lys-Phe, was compared to the method of entrapment, employing the use of polyene antibiotics.
4.5.2. THE SYNTHESIS OF L-LYSYL-L-(14C)-PHENYLALANINE. 

(14C-LYS-PHE).

INTRODUCTION.

The synthesis of 14C-Lys-Phe was carried out using a protocol kindly supplied by G.D.Searle and Co.Ltd(Dyer et al., 1981).

L-lysine was protected as the dicarbobenzoxy derivative and was coupled with phenylalanine, which was also protected as the benzyl ester p-toluene sulphonate salt, via a mixed anhydride reaction using iso-butyl chloroformate in toluene, with triethylamine as a base giving protected Lys-Phe.

The deprotection of the radiolabelled dipeptide was carried out via a catalytic transfer hydrogenation in methanol, using a 10% palladium on carbon catalyst and formic acid as hydrogen donor. The product formed, was the formate salt of Lys-Phe which was converted to the dihydrochloride by the addition of 2 mole equivalents of hydrochloric acid.

Carbon-14-L-phenylalanine was obtained from Amersham International, Amersham, with an activity of 496mci/mmol. All other chemicals were obtained (unless otherwise stated) by Sigma Chemical Co, Poole.
EXPERIMENTAL PROCEDURE.

STAGE 1. PROTECTION OF CARBON-14-PHENYLALANINE TO FORM THE BENZYL ESTER,p-TOLUENE SULPHONATE.

A mixture of L-phenylalanine (6.94g), p-toluene sulphonic acid (9.70g), benzyl alcohol (43.89g) and benzene (150ml) was refluxed with stirring for 5 hrs. All the solid was in solution after about 20 mins. The water was removed by azeotropic means.

Carbon-14-L-phenylalanine was added to the flask; the amount determined by its activity. Since a certain amount of radioactivity would be lost during the synthesis, a high activity was required, therefore 10 micro-litres was added.

After 5 hours, the benzene was removed on a rotary evaporator at 45°C followed by trituration of the resulting oil with ether (150ml). This resulted in the formation of a white crystalline solid which was filtered, washed again with ether (80ml) and dried in air. The crude product was recrystallized from a mixture of ethanol (40ml) and ether (40ml), and then washed under reflux for 15 mins with ether (150ml). The product was then dried overnight at room temperature.

The purity of the product was determined by thin-layer chromatography (TLC), using cellulose plates and developed in a solvent system of n-butanol: HCl: pyridine: water in a ratio of 60: 12: 40: 48. The plate was sprayed with ninhydrin (B.D.H. Chemicals, Poole), and heated which produced a single spot. Purity was also confirmed by its melting point.
STAGE 2. PROTECTION OF L-LYSINE TO FORM ALPHA,

E-DICARBOBENZOXY-L-LYSINE.

A stirred solution of L-lysine monohydrochloride (5.48g) in 2N sodium hydroxide (30ml), was cooled to 5°C. From two separate dropping funnels benzyl chloroformate (10.24g) and 4N sodium hydroxide (15ml) was added simultaneously at such a rate that the addition lasted 50 mins. The temperature of the reaction mixture was kept below 5°C, and the pH below 11.5. A white emulsion was formed which was stirred overnight.

The reaction mixture was washed with ether (120ml), cooled overnight and acidified by the slow addition of concentrated HCl (4.3ml) using congo red as indicator. Extraction was achieved with chloroform (3x60ml), followed by drying over sodium sulphate. The solvent was removed on a rotary evaporator, leaving a colourless oil which was dried overnight at ambient temperature.

Purity was determined by TLC on silica plates using n-butanoic acid as the solvent. The plate was treated with iodine vapour and a single spot was produced which was visible under ultra-violet light at 254nm.
STAGE 3. COUPLING REACTION TO FORM ALPHA,
E-DICARBOBENZOXY-L-LYSYL-L-CARBON-14-
PHENYLALANINE BENZYL ESTER.

A solution of alpha,e-dicarbobenzoxy-L-lysine(3.97g) and triethylamine(0.97g) was prepared, and to it, iso-butyl chloroformate(1.49g) was added over a period of 15 mins, keeping the temperature between -5 and 0 °C. The resulting jelly-like mixture was stirred for 30 mins at 0 °C and then a slurry of L-phenylalanine,p-toluene sulphonate(4.10g) in chloroform(47ml), was added quickly in one portion and the clear solution was stirred at ambient temperature overnight.

The reaction mixture was washed with 4% sodium bicarbonate solution(2×90ml), followed by 2% HCl(2×90ml) and finally with water (2×32ml). It was then dried over sodium sulphate and the solvent removed on a rotary evaporator. A white solid was formed which was recrystallized from a mixture of hexane(35ml) and benzene(70ml). The white crystalline solid was washed with hexane(20ml) and dried at ambient temperature under vacuum.

Purity was determined by TLC using silica plates and developed with n-butanoic acid. A single spot was formed which was visible under ultra-violet light at 254nm. The melting point was also determined (theoretical value: 139-142 °C).
STAGE 4. DEPROTECTION TO FORM L-LYSYL-L-PHENYLALANINE DIHYDROCHLORIDE.

A 10% palladium on charcoal catalyst (2.5 g) was placed in a flask and purged with nitrogen gas. A solution of formic acid (4.4%) in methanol (150 ml) was added slowly, followed by a slurry of the protected dipeptide (2.5 g in 100 ml methanol).

After stirring at room temperature for 45 mins, the reaction mixture was filtered and then evaporated to dryness on a rotary evaporator resulting in the formation of a clear oil, which was the crude formate salt of the dipeptide. The oil was dissolved in water (20 ml), and 1N HCl (7.68 ml) was added. The emulsion was evaporated to dryness on a rotary evaporator, leaving a very pale yellow oil, which was dissolved in water (30 ml) and freeze-dried. At this stage, difficulty arose in crystallizing the oil and so an equal weight of "cold" Lys-Phe (Sigma Chemical Co.) was added and stirred vigorously. This proved to be successful.

Purity was determined again by TLC, using silica F-254 plates, and developed using a solvent system of propan-2-ol: concentrated ammonia, in a ratio of 70:30. A single spot was formed after spraying with ninhydrin.

The activity of the product was determined by liquid scintillation counting, using Instagel (Packard, Illinois, U.S.A.) as the cocktail and counted on a scintillation counter (Beckman, LS-230 U.S.A.).
SCHEME.

L-PHENYLALANINE (I) →
+PhCH₂OH
+p-TOLUENE SULPHONYL CHLORIDE
+BENZENE

L-PHENYLALANINE BENZYL ESTER, p-TOLUENE SULPHONATE (III) →

L-LYSINE (II) →
+PhCH₂OCOC₁
+NaOH
+WATER

ALPHA,E-DICARBOBENZOXY-L-LYSINE (IV) →

ALPHA,E-DICARBOBENZOXY-L-LYSYL-L-(14-C)-PHENYLALANINE BENZYL ESTER (V) →
+1.Pd/C, HCO₂H, MeOH
+2.HCl

L-LYSYL-L-(14-C)-PHENYLALANINE DIHYDROCHLORIDE (VI)
4.53. THE PREPARATION OF ERYTHROCYTES ENCAPSULATED WITH RADIOLABELLED LYS-PHE FOR LIQUID SCINTILLATION COUNTING.

When counting the cells, it was important that all the haemoglobin was removed and that the resulting solution to be counted was free from any colour. Two such methods were employed:

METHOD A. CHLOROFORM/ETHANOL EXTRACTION.

This method was based on the work of Concetti et al. (1976).

1) 0.1ml of the encapsulated preparation was haemolysed with an equal amount of water.

2) 0.125ml of ice-cold chloroform was added slowly, followed by 0.25ml of ice-cold ethanol. The contents of the tube were vortexed for 3 mins, ensuring that the tube remained cold.

3) 0.8ml of ice-cold water was then added, and was further vortexed for 10 mins. At this point the haemoglobin had settled to the bottom of the tube, leaving a milky supernatant.

4) The tube was then centrifuged at 4xC for 10 mins at 30,000g using an ultra-centrifuge (MSE-Superspeed 50), which resulted in a clear supernatant.
5) The radioactivity of the supernatant was then counted in 10ml Instagel using a liquid scintillation counter. To reduce chemiluminesence, samples were counted after 3 hours. The counting efficiency was determined using an internal standard. (See section 4.54.)

**METHOD B. EXTRACTION USING TISSUE SOLUBILISER.**

This method incorporated the use of a solubiliser which digested the blood, followed by bleaching the sample to remove the colour.

1) 0.5ml of the encapsulated preparation was used for counting, and was placed into a 22ml glass liquid scintillation vial which had a polyethylene-lined screw cap.

2) 12 times (the volume of blood sample) of tissue solubiliser (Scintran, B.D.H. Chemicals, Poole) was then added and the mixture was swirled gently.

3) The vial was capped tightly and incubated in a shaking water bath at 60°C for 1 hr.
4) The vial was removed from the water bath and 0.5ml of hydrogen peroxide (30%) was immediately added. Unlike human blood, rat blood does not foam during the addition of hydrogen peroxide if it is added all at once to a warm mixture.

After the addition of the peroxide, the digest underwent a series of colour changes from brown to purple to yellow; the yellow tinge being proportional in intensity to sample volume.

5) The vial cap was loosened and was incubated for an additional 30 mins. The contents were cooled, to which 15ml Instagel was added and the vial was shaken vigorously.

6) 0.5ml of 0.5N HCl was added, and the vial was shaken again, left to equilibrate for a further 30 mins and counted. The HCl was added to reduce the chemiluminesence. The order of addition of the cocktail and the HCl was important; such that they were not combined but dispensed as a single reagent.
4.54. THE DETERMINATIONS OF COUNTING EFFICIENCIES.

In liquid scintillation counting, counting efficiency can be determined by three basic methods:

a) By an internal standard.

b) By the samples channel ratio method.

c) By the external standard ratio method.

The method adopted for the experiments described, was the internal standard method (SPIKE METHOD). The sample to be counted was first counted and the counting rate determined. An aliquot of known radioactivity (a standard) was added to the vial and again counted. This count would therefore be similarly affected by the quenching agent and so the counting efficiency was determined by the following formula:
C.P.M.(sample) = C.P.M.(spike+sample) - C.P.M.(spike)
= D.P.M.(spike) x EFF(spike)

Assume that EFF(spike) = EFF(sample)
Therefore, D.P.M.(sample) = C.P.M.(sample)
           EFF(sample)
= C.P.M.(sample)
           EFF(spike)

Where C.P.M. = counts/min
D.P.M. = disintegrations/min
EFF(sample) = counting efficiency of the standard.

The internal standards used were tritium-labelled hexadecane and carbon-14 labelled hexadecane. (Amersham International, Amersham) Counting efficiencies were obtained using both the chloroform/ethanol and digestion extraction methods. The method which gave the highest value was then used for assaying Lys-Phe.
4.55. DETERMINATION OF THE LOCATION OF LABELLED LYS-PHE IN ERYTHROCYTES.

In order to determine whether Lys-Phe was entirely encapsulated within the erythrocyte, or whether it was attached to the erythrocyte membrane, the following experiment was conducted:

1) Blood was removed from a rat by cardiac puncture, centrifuged, and the plasma removed.
2) The packed cells were divided into two portions. The first portion was treated according to the encapsulation procedure, and assayed by the chloroform/ethanol extraction method and counted in a liquid scintillation counter (see section 4.53). The percentage encapsulation was thus calculated.
3) To the second portion of packed cells, the same amount of drug (made up in isotonic Hanks medium) was added and incubated in a shaking water bath at 37°C for approximately 2 hrs (i.e. the approximate duration of an encapsulation experiment). The cells were then washed twice with 5 ml of isotonic Hanks medium and the erythrocytes assayed as before.
4) The amount of radioactivity found in the cells from stage 3 indicated the amount of drug attached to the cells, and therefore this value was subtracted from the value obtained from stage 2, to give the actual percentage encapsulation. The experiments were performed using both carbon-14 and tritiated Lys-Phe, made up in isotonic Hanks medium, to give a stock solution of 40mM.
4.56. THE INCORPORATION OF INCREASING AMOUNTS OF LYS-PHE IN RAT ERYTHROCYTES.

In order to establish the optimum amount of Lys-Phe required to produce the maximum encapsulation, a series of tubes with increasing quantities of Lys-Phe were set up as described:

1) Cells were subjected to the encapsulation procedure up to the point of drug addition. Increasing volumes of Lys-Phe (40 mM) were added onto the haemolysate layer to different tubes containing 1 ml of packed cells.

2) The cells were then resealed using 10× Hanks medium (the volume calculated by the formula described in section 4.42.).

3) After washing the cells twice with isotonic Hanks medium, the cells were assayed by extracting using the chloroform/ethanol method and counted by liquid scintillation.

4) Both radioisotopes of Lys-Phe were used, and a graph of volume of drug added against percentage encapsulation was produced. The amount of drug that was attached to the membrane in each sample was determined in the same manner as described in section 4.55.
4.57. THE RELEASE OF LABELLED LYS-PHE FROM ERYTHROCYTES THROUGH DIALYSIS SACS.

The membrane of a standard dialysis sac was used as an analogue of the erythrocyte membrane to elucidate if slow release through the cell membrane was a possible mechanism for the release of Lys-Phe from encapsulated erythrocytes.

1) Standard dialysis tubing (Visking, Medicell International Ltd, London) was used for these experiments. Small sacs 100mm x 10mm were made and sealed at one end.

2) A 0.5ml portion of the material under test was added to the sac, and suspended in 25ml of isotonic Hanks medium at a depth of 50mm using McCartney bottles as containers.

3) The sacs were transferred to fresh medium every 30 mins and the experiments terminated after 3 hrs. Each preparation was tested in 5 replicate sacs. A 1% solution of methylene blue was used as a standard. By coincidence, it had a molecular weight similar to that of Lys-Phe (374 for methylene blue compared with 329.9 for Lys-Phe). The amount of methylene blue released from the sacs was measured by its absorbance at 664nm (Pye Unicam SP500 spectrophotometer).
Three different sacs were prepared as followed:
  a) Erythrocytes encapsulated with Lys-Phe.
  b) Free Lys-Phe.
  c) Untreated erythrocytes + Lys-Phe.

4) The surrounding medium was then counted for its radioactivity as before (i.e. 1 ml of medium added to 10 ml Instagel), and graphs of dialysis time against amount of drug released in the medium were constructed.
4.58. T.L.C. EXAMINATION OF ENCAPSULATED ERYTHROCYTES CONTAINING LYS-PHE.

In order to establish whether the dipeptide remained intact (i.e. it was not broken up into single amino acid residues) after encapsulation, and also after leakage from the cell, TLC of the supernatants was employed.

1) Lys-Phe was encapsulated into erythrocytes as before and the haemoglobin extracted using the chloroform/ethanol method.

2) Extracts from stage 1 were concentrated on Merck Kieselgel F 254 plates, using 50 micro-litres spots at a time, until a total volume of 50 micro-litres was applied. The plates were developed using a solvent system of propan-2-ol and 0.1% ammonia in a ratio of 70:30. Standard extracts were made up in chloroform/ethanol and likewise applied to the plates.

3) The plates were run for about 2 hrs, after which, they were dried by passing hot air over the plates. The plates were sprayed with ninhydrin, and examined for a single spot, indicating the presence of the intact dipeptide.

4) The above procedure was repeated for samples, that had leaked out from the cells (section 4.57) to determine if the dipeptide survived intact after diffusion through the cell membrane.
4.59. THE ENCAPSULATION OF LYS-PHE INTO INTACT SS-RED BLOOD CELLS.

In order to determine whether Lys-Phe, encapsulated into SS-RBC, would inhibit sickling in situ, the dipeptide was incorporated into such cells and then examined under the electron microscope.

1) Homozygous blood was obtained from a patient of West Indian origin (Birmingham General Hospital, Birmingham).

2) After centrifugation at 1600g for mins, the plasma and buffy coat was removed and the packed cells were washed twice with 5ml of isotonic phosphate buffer.

3) Trituated Lys-Phe (40mM) was then encapsulated into a 1ml portion of packed cells using the method described in section 4.42.

4) Encapsulated and untreated cells were then deoxygenated with nitrogen at 37°C for 20 mins and then fixed in a 2.5% glutaraldehyde solution (buffered at pH 7.4 with deoxygenated Sorensen's phosphate buffer) in a ratio of 1:10 for 1 hr. The cells were then washed three times with Sorensen's phosphate buffer.
5) Using a small pipette, one drop of buffer containing erythrocytes was applied to an aluminium carrier of an electron microscope. After a few seconds, excess water was removed from the carrier with filter paper and the residue was dried with warm air.

6) The carrier was then sputtered with gold for 3 mins (3 x 1 min bursts), leaving time between each burst to ensure that the sample did not become too hot, and therefore, would cause damage or denaturation.

7) At this stage, samples could be stored in a desiccator until photomicrographs could be obtained. Photomicrographs were taken using a Cambridge Stereoscan S150 scanning electron microscope. Exposure time was 60 secs. Photographs were also taken under normal tungsten illumination, and cell counts were taken. The results were expressed as a percentage of sickled cells to unsickled cells for both untreated and cells treated with Lys-Phe.

8) An estimation of the amount of tritiated Lys-Phe encapsulated into the SS-RBC was assayed using the chloroform/ethanol extraction method (section 4.53.), and the radioactive content counted by liquid scintillation.
4.6. ERYTHROCYTE ENTRAPMENT OF LYS-PHE BY AMPHOTERICIN-B WITHOUT HAEMOLYSIS.

Polyene antibiotics, such as amphotericin-B, damage microorganisms by increasing the permeability of their membranes to metabolites and ions (Deuticke et al., 1973). This property was exploited in this experiment when amphotericin-B was used to load erythrocytes with Lys-Phe.

1) Blood was removed from male Wistar rats by cardiac puncture, centrifuged at 600g for 10 mins and the plasma and buffy coat removed.

2) Using 1ml portions of packed cells, they were washed 4 times with 0.9% NaCl, followed by 2 washes with an isotonic sucrose medium (containing 250mM sucrose, 5mM KCl, 5mM magnesium chloride, 2mM potassium phosphate buffer, pH 7.4 and 10mM glucose).

3) The cells were suspended in 5ml sucrose medium, and then pre-incubated for 5 mins with various concentrations of amphotericin-B (stock solution = 10 micro-grams/ml). The pharmaceutical preparation was used i.e. in combination with sodium deoxycholate (Sigma Chemical Co., Poole). Tritiated Lys-Phe was then added to the suspension. Both amphotericin-B and Lys-Phe were dissolved in sucrose media just before use.
4) The suspension was incubated at 37°C for 30 mins with gentle shaking. The entrapped erythocytes were collected by centrifugation at 3000g for 5 mins and washed 3 times with isotonic phosphate-buffered saline containing 2mM EDTA. EDTA diminishes the susceptibility of cells to lysis after treatment with drugs.

5) The amount of Lys-Phe entrapped within the erythocytes was determined as described earlier (section 4.53.), and TLC of the supernatants from the extractions were performed using the method described in section 4.58. This was to establish whether the amphotericin-B had not broken down the dipeptide into single amino acid residues.

6) In vivo survival of entrapped erythocytes was monitored by labelling the cells with FITC and reinjecting them into the rat (see section 4.44.).
4.6. THE RELEASE OF LYS-PHE FROM ERYTHROCYTES AFTER ENTRAPMENT USING AMPHOTERICIN-B.

1) Lys-Phe was entrapped into erythrocytes using amphotericin-B to facilitate its entry. (Method described in section 4.6.)

2) A 0.5ml portion of the entrapped erythrocytes was added to a dialysis sac (10×100mm) and suspended in 25ml sucrose medium.

The following sacs were prepared:

a) Free tritiated Lys-Phe
b) Entrapped erythrocytes containing tritiated Lys-Phe
c) Untreated erythrocytes (incubated) with tritiated Lys-Phe
d) Free tritiated Lys-Phe + amphotericin-B

3) The sacs were transferred to fresh medium every 30 mins and the experiments terminated after 3 hrs. Each preparation was tested in 5 replicate sacs.

4) A 1ml portion of each sample was added to 10ml Instagel, and counted on a liquid scintillation counter.

5) The results of this experiment were compared with those obtained from the release rate studies involving erythrocytes subjected to the encapsulation procedure.
4.7. **THE ENCAPSULATION OF UROGASTRONE INTO INTACT ERYTHROCYTES.**

4.7.1. **INTRODUCTION.**

A similar protocol to Lys-Phe was adopted with the polypeptide urogastrone. Initially the optimum loading value of urogastrone was established and the in vivo survival of circulating erythrocytes was monitored by FITC labelling. It was decided to initially iodinate the urogastrone, thus enabling the peptide to be assayed, and also to study the in vitro release from erythrocytes.

Pharmacological activity of encapsulated preparations was determined using two biological assay systems. Firstly, the perfused rat stomach preparation was used as a measure of the continuous acid secretion and secondly the pylorus-ligated rat was employed to give a measure of the spontaneous acid secretion.

All materials, unless otherwise stated, were obtained from Sigma Chemical Co., Poole. Urogastrone was also supplied from Sigma in a crude form obtained from human pregnancy urine.
4.72. THE RADIOIODINATION OF UROGASTRONE.

A variety of methods are available for the radioiodination of peptides and proteins suggesting that no single method has been found to be completely satisfactory. Protein iodination methods can be broadly divided into two groups; direct methods—those in which radiiodine is directly incorporated into tyrosine residues of the protein chain, and conjugation methods—those in which a radiiodinated moiety is conjugated to a specific sidechain of the protein.

Generally, the direct iodination methods have the advantage of being more straightforward, involving a single stage reaction in which the protein is reacted with radiiodide. Conjugation labeling methods are more complex, and iodination yields are lower.

In order to obtain iodinated preparations of high specific activities without the use of large amounts of radiiodide, one must either use small masses of protein for labelling, or label larger amounts of protein and use some method for separating iodinated from non-iodinated material.
Materials for the direct iodination of proteins and peptides include: a) the iodine monochloride method, b) the chloramine-T method, c) alternative chemical oxidation methods, d) electrolytic iodination and e) enzymatic iodination.

It was decided to employ the chloramine-T method to iodinate urogastrone. This method is the most widely used method for the radioiodination of small masses of protein to give high specific radioactivities for use as tracers. Na $^{125}$I was oxidized by chloramine-T in the presence of the peptide to be labelled, with the subsequent incorporation of $^{125}$-iodine into the tyrosine residues of the protein in high yields. Excess chloramine-T was reduced by the addition of sodium metabisulphite, and free iodine was reduced to iodate.

After the addition of an excess of unlabelled NaI or KI to act as a carrier for the high specific activity $^{125}$-iodide, and a protein-containing buffer to act as a carrier for the labelled protein, labelled protein was separated from unreacted iodide by gel filtration.
PROCEDURE.

A. IODINATION.

1) A 1.0mci aliquot of Na 125-I solution (Amersham International, Amersham) was dispensed into a polystyrene tube. 10 micro-litres of 0.25M phosphate buffer, pH 7.5 was added to buffer the iodide solution.

2) The following solutions were added in rapid succession, whilst continuously agitating the reaction mixture with a small glass-covered magnetic stirrer. (All the solutions were freshly prepared in 0.05M phosphate buffer, pH 7.5.)

A: 10 micro-litres (1.5mcg) urogastrone solution
B: 10 micro-litres (50mcg) chloramine-T solution
C: 100 micro-litres (120mcg) sodium metabisulphite solution

3) The volume of the solution was made up to 1.0ml with KI (2mg/ml) solution and the iodination tube was counted in a L.P.3. tube (Luckham Ltd, West Sussex). This contained a known amount of 125-I iodide (the number of mci was calculated from the known volume of Na 125-I taken), and represented the count of the total radioactivity taken for the iodination. All subsequent radioactive measurements were then made in identical tubes in a volume of 1.0ml so that they all were compared directly.
B. Separation of Labelled Urogastrone from Unreacted Iodide.

4) The reaction mixture was transferred to a 0.9*27cm column (Pharmacia), packed with Sephadex G-25 resin, which had been previously saturated with 0.5ml bovine serum albumin (100mg/ml) in phosphate buffered saline (containing 50mM phosphate-buffered saline, pH 7.5, 77mM NaCl and 0.1mg/ml thimerosal). This was to minimize the adsorption of the very low concentrations of labelled urogastrone to be separated.

5) The iodination reaction mixture was quantitatively transferred to the prepared column and eluted with phosphate-buffered saline. 1.0ml fractions were collected into L.P.3. tubes of the same dimensions as the iodination reaction tube. The column was run until both the urogastrone and the 125-I iodide peaks had been eluted, and the radioactivity in all the fractions was measured.
C. CALCULATION OF YIELD AND SPECIFIC ACTIVITY.

If a "protein-free" radiiodination was carried out, the original radioactivity could be quantitatively recovered in the iodide peak eluted from the gel filtration column. Hence if, in a protein containing iodination reaction, the recovery of radioactivity from the column was not quantitative, the lost counts must represent labelled protein lost by adsorption.

The radioactivity incorporated into the urogastrone was therefore calculated as:

radioactive counts in protein = original total counts - counts in iodide peak

As the original radioactivity was determined by carefully measuring a known volume of a solution of stated radioactive concentration, the counts incorporated into the urogastrone was converted into units of radioactivity (micro-curie). As the original mass of urogastrone was known, the specific activity of the resulting labelled urogastrone was calculated.

The mass of urogastrone recovered in each of the fractions of labelled urogastrone eluted from the column was calculated and hence a tracer solution was prepared containing a known concentration of labelled urogastrone.
6) The urogastrone fractions containing the greatest number of counts were diluted 1:10 with a standard diluent and 1ml aliquots were frozen in liquid nitrogen and stored at -35°C.

**4.73. THE ENCAPSULATION OF LABELLED UROGASTRONE INTO INTACT ERYTHROCYTES.**

Using the iodinated urogastrone, it was possible to estimate the amount of peptide internalized within the erythrocytes by simply counting in a gamma counter. FITC labelled cells, injected back into the rat enabled the in vivo survival of circulating cells to be monitored.

1) Blood was removed by cardiac puncture from male Wistar rats, centrifuged and the plasma and buffy coat removed.

2) 1ml portions of packed cells were then subjected to the encapsulation procedure as described in section 4.42. A stock solution of urogastrone (10 micro-gram/ml) containing a known amount of the tracer solution was prepared. Urogastrone was added to a series of tubes in increasing volumes.
3) The cells were resealed with a calculated volume of 10\*Hanks medium and then washed twice with 5ml isotonic Hanks medium. A further 1ml portion of packed cells was incubated with various amounts of urogastrone. This was to determine the amount of urogastrone attached to the erythrocyte membrane.

4) In vivo survival of drug-loaded erythrocytes in rats was monitored as described in section 4.44.

5) Samples to be assayed were transferred to L.P.3. tubes and counted in a gamma counter (Gamma Set 500 ICN Instruments, Belgium), and the percentage encapsulation was calculated.
4.74. THE RELEASE OF LABELLED UROGASTRONE FROM
ERYTHROCYTES THROUGH DIALYSIS SACS.

The membrane of a standard dialysis sac was used as an
analogue of the erythrocyte membrane to elucidate if slow
release through the cell membrane was a possible mechanism
for the release of urogastrone from erythrocytes.
1) The method adopted for the release of Lys-Phe from
erythrocytes (see section 4.57.) was employed in this
experiment.

2) The following dialysis sacs were prepared:
   a) Free urogastrone (5 micro-grams).
   b) Erythrocytes encapsulated with 125-I-urogastrone.
   c) Untreated (incubated) erythrocytes + 125-I-urogastrone.
3) The surrounding medium was counted for its
   radioactivity. 1ml of the medium was transferred into a L.P.3.
tube and counted in a gamma counter.
4) Graphs of dialysis time against the amount of labelled
   urogastrone released into the medium were constructed.
4.75. THE ASSAY OF UROGASTRONE USING THE PERFUSED RAT STOMACH.

The preparation of the perfused rat stomach in which acid secretion was electrometrically recorded was first employed by Ghosh and Schild (1958). The method used in this experiment was based on the reperfusion of the rat’s stomach developed by Smith et al. (1970), where an "integrated" record of total acid secreted during the test period was measured as compared to the "differential" procedure adopted by the former workers. The assay design, described by Lawrence et al. (1971), expressed the activity of urogastrone in terms of the log-response curve of carbachol in that animal.

PROCEDURE.

1) Male Wistar rats of 180-330g were starved for 16-24 hrs with free access to water, and anaesthesia was then induced by an intraperitoneal injection of pentobarbitone sodium (0.44ml/kg). (Sagatal, May and Baker, Dagenham).

2) The body temperature was maintained at 30°C by a 25W lamp under the table on which the animal lay and an overhead 40W light.
OPERATIVE TECHNIQUE.

3) The trachea was exposed and cannulated.

4) A polythene tube of 2.5mm external diameter (Portex Ltd, Hythe) was passed down the oesophagus and tied in place in the neck, excluding the vagal nerves.

5) A fine polythene cannula (Portex Ltd, Hythe) was used to cannulate the femoral vein which was tied secure.

6) The abdomen was opened through the linea alba and a glass cannula passed through an incision in the duodenum approximately 3cm from the pylorus, and gently slipped into the stomach, care being taken to avoid handling the stomach. The cannula was 6mm in external diameter with a waist 2cm from the tip, which was 4.5mm in diameter, which snugly fitted the pylorus. The intragastric portion of the cannula contained numerous perforations. No ligature was required round the pylorus (part of the cannula) thus leaving the gastric blood supply intact, but the cannula, was tied in place with a ligature round the duodenum.

7) Any gastric contents were washed out with warm saline as a clean, freely-draining stomach was essential for good results.
GASTRIC PERFUSION SYSTEM.

(Shown diagrammatically in Fig. 4.)

8) The stomach was continuously perfused with 20ml of a propionic- succinic acid buffer, pH 5.5 in normal saline at 30°C, recirculated at a rate of 3ml/min by means of a Watson-Marlow roller pump; the gastric effluent dripping back into a reservoir.

9) A stock solution of buffer was prepared by dissolving 0.5 mole propionic acid (37g), 0.5 mole succinic acid (59g) with 1 mole solid sodium hydroxide (40g) in water and diluting to 1 litre. The final pH adjustment to pH 5.5 was made with a glass electrode using N NaOH or N HCl. From this solution, fresh buffer was made up for each experiment by diluting 5ml to 1 litre, or for maximal sensitivity, 3.33ml to 1 litre. The buffer was changed after each test to reduce changes due to gastric absorption.
EXPERIMENTAL PROCEDURE.

A pH meter (Corning-EEL Model-5), connected to a recorder (Smiths Servoscribe RE.511.20) was used to record the acid output. The chart was calibrated so that a full scale deflection equalled 1.0 pH unit and, knowing the volume and concentration of the buffer, the acid secretion could be calculated in micro-equiv hydrogen ions from the change in pH. (Chart speed = 600mm/hr.)

10) The basal acid output was recorded for 15 mins before injecting the gastric stimulant (carbachol). The stomach was washed with warm saline and perfusion was begun using fresh buffer. Carbachol (0.5 micro-grams) was given intravenously and the response recorded over 40 mins to check the sensitivity of the preparation. The first dose tested gave inconsistent results and so was disregarded. The buffered reperfusion solution was then changed and the routine repeated, thus the experiment started with the second dose administered.

11) Urogastrone preparations were injected 5 mins before stimulant carbachol doses.
The inhibitory effect of urogastrone was measured in terms of a logarithmic "carbachol index" (C.I.), defined as follows:

\[ C.I. = 0.3 \times \frac{C(1) - C(0)}{C(1) - C(2)} \]

where \( C(1) \) = response to standard dose of carbachol, \( C(2) \) = response to half the standard dose of carbachol and \( C(0) \) = response to standard dose of carbachol preceded by a dose of urogastrone.

Expressing inhibition in terms of the dose-response curve of carbachol gave more consistent results than expressing it simply in terms of pH changes.

12) A dose response curve of carbachol using a range of concentrations of free urogastrone was constructed, followed by the testing of encapsulated preparations on the same system. The "carbachol index" for both free and encapsulated urogastrone was also calculated.
13) As a standard drug inhibitor, cimetidine was administered intravenously at a dose of 240mg/kg and the assay commenced as before. (Cimetidine was a gift from S.K.&F.Labs, Welwyn Garden City, Herts.)

14) "Sham" encapsulated preparations (i.e. erythrocytes encapsulated with 0.1% saline), were also tested to determine whether the urogastrone-loaded erythrocytes themselves, or simply the injection of erythrocytes into the rat inhibited the acid secretion.
FIG. 4. DIAGRAM OF REPERFUSION SET UP.
4.76. THE ASSAY OF UROGASTRONE USING THE PYLORUS-LIGATED (SHAY RAT) PREPARATION.

The pylorus-ligated rat preparation was first described by Shay et al. (1945), hence known as the Shay rat. This preparation was used to measure the spontaneous gastric acid secretion whereas the perfused rat stomach was a measure of the continuous secretion.

1) Male Wistar rats weighing between 80 and 170g were starved from periods between 24-72 hrs, with free access to water.

2) The animals were anaesthetized with ether and the abdomen opened by a midline incision. A cotton thread was placed around the pylorus, taking care to avoid the gastro-duodenal artery.

3) After closure of the abdominal incision, the stomach was washed by introducing 4ml of warm isotonic saline orally, and sucking the liquid out again. If less than 4ml were recovered, the animal was discarded since this indicated the presence of food or faeces in the stomach. The stomach was then left empty and the wound sprayed with colloidan (Nobecutane, Astra Ltd, Watford).
4) Either free, or encapsulated urogastrone preparations, were administered 45 mins before the pylorus was ligated, via the femoral vein which had previously been cannulated.

5) 2-6 hours after ligation the animal was killed; the oesophagus ligated and the stomach removed. An opening was made along the greater curvature and the contents removed, centrifuged and the volume of the gastric juice recorded. The acidity was determined by volumetric titration using phenolphthalein as indicator.
4.8. POSSIBLE STORAGE SYSTEMS FOR DRUG-ENCAPSULATED ERYTHROCYTES.

4.8.1. INTRODUCTION.

When red cells are stored in the liquid state, they progressively lose their ability to survive in vivo. After relatively brief periods of storage, the majority of cells are still capable of normal survival, but a minority are destroyed within the first 24 hrs after injection. After longer periods of storage, all the cells show some reduction in survival although, again, the rate of destruction in the first 24 hrs is much greater than it is subsequently.

For encapsulated preparations to become an actual commercial proposition clinically, a suitable method of storage would have to be developed. This storage system should ideally be robust, easily executed and most important, it should withstand transportation.
It was decided that a system of suspending the red cells in either a gelatin or agarose gel would be investigated. Initially the nature and the properties of the gel was established, and after extended periods of storage, the in vivo survival of labelled cells was monitored. In vitro viability tests were also conducted on stored erythrocytes. Finally, the effect of freeze-drying on the survival characteristics of red cells was also investigated.

4.82. THE STORAGE OF DRUG-ENCAPSULATED ERYTHROCYTES ON GELATIN GELS.

A. THE DETERMINATION OF THE PHYSICAL PROPERTIES OF THE GEL.

A suitable gel for the suspending of red cells should ideally have the following properties: firstly, the gel should set relatively quickly once cooled to 4°C and secondly, when required, the gel should liquefy easily (i.e. within minutes) and then be separated from the red cells by centrifugation.

A "soft bloom" gelatin was used as this would cause minimal stress to the erythrocytes.
1) Gelatin (60 Bloom, Sigma Chemical Co, Poole) obtained from swine skin was used to prepare the gels.

2) A series of gelatin solutions (1%, 2%, 3% and 4%) were made up in Hanks medium, by gently warming the Hanks medium and gelatin, with constant stirring, until the gelatin was completely dissolved. It was important that the solutions were not overheated since this would result in a reduction in the strength of the gel.

3) 5ml portions of the gelatin solution were then transferred to a series of test tubes and were then left to set in the refrigerator at 4°C, ensuring that the time taken for them to set was recorded.

4) Upon setting, the gels were then incubated at 37°C and the time taken for them to liquefy was also recorded. Trypsin (1mg/ml) was added to certain samples as a means of decreasing the liquefying time. The data was collected and the most suitable gel was selected for testing the storage of erythrocytes.
B. THE IN VIVO SURVIVAL OF DRUG-ENCAPSULATED ERYTHROCYTES
AFTER STORAGE ON GELATIN GELS.

The gel selected from the previous experiment was used to store erythrocytes for extended periods, after which time, the in vivo survival was monitored of reinfused cells. Before storage, the cells were also incubated with a variety of cytoplasmic constituents in an effort to increase the storage and in vivo survival time.

1) Blood was obtained by cardiac puncture from male Wistar rats and subjected to the encapsulation procedure as described in section 4.42.

2) The erythrocytes were then brought to 37° C and incubated with various cytoplasmic constituents for 3 hrs.

The following constituents were made up in isotonic Hanks medium and added to the cells in 25 micro-litre volumes. (All the nucleotides were purchased from Sigma Chemical Co, Poole.):

a) 20mM Adenosine.
b) 5mM Inosine.
c) 5mM Glucose.
d) 25mM Guanosine.
3) A 3% "soft bloom" gelatin solution in isotonic Hanks medium was prepared and equilibrated for 1 hr with a mixture of 95% oxygen and 5% carbon-dioxide. Erythrocytes were then suspended and set in the form of a "slope", and the gels stored for up to 14 days.

4) After prolonged storage, the gels were gently warmed and then centrifuged so as to separate the erythrocytes from the gelatin.

5) The cells were then labelled with FITC and returned to the rats' circulation via the femoral vein, and cell counts obtained from the tail vein were taken at regular intervals. (See section 4.45.)
4.83. ESTIMATION OF THE LIFE-SPAN OF ERYTHROCYTES IN VIVO

USING THE RADIOACTIVE CHROMIUM METHOD.

The in vivo survival monitoring of drug-encapsulated erythrocytes described earlier employed the use of FITC as the means of labelling the cells (section 4.43). However, its main disadvantage was that the intensity of fluorescing cells diminished very quickly, and after about 7 days, there was great difficulty in visualizing labelled cells under the microscope.

A more accurate method involves the use of the radioisotope, chromium-51. This isotope has a half-life of 27.8 days, and after passing through the surface membrane of the red cells, the labelled sodium chromate is reduced to the trivalent form which binds to protein, preferentially to the beta-polypeptide chain of haemoglobin (Dale and Lewis, 1984). Chromium is toxic to red cells, probably by its oxidizing action; it inhibits glycolysis when present at a concentration of 10 micro-grams/ml of red cells or more and blocks glutathione reductase activity at 5 micro-grams/ml. Blood should thus not be exposed to more than 2 micro-grams of chromium/ml of packed red cells.
PROCEDURE.

1) 0.5 micro-curies of sodium-51-chromate/kg body weight (New England Nuclear, Edinburgh, Scotland) was added to 1 ml of drug-encapsulated erythrocytes, and incubated at 37°C for 15 mins. The mixture was washed twice with 0.9% saline.

2) The labelled cells were injected back into the rat via the femoral vein (section 4.43.) and a tail vein sample taken after 5 min. The radioactivity of this sample provided the baseline. The next sample was taken 24 hrs later, followed by another sample at day 1, 2, 4, 7 and thereafter, until at least half the radioactivity had disappeared from the circulation.

The percentage survival of chromium-51 on any day (t) was estimated by comparing the radioactivity of the sample taken on that day with that of the day (0) sample.

Thus, chromium-51 survival on day (t) =

\[
\frac{\text{C.P.M. / ml of blood on day } t}{\text{C.P.M / ml of blood on day } 0}
\]

No adjustment was necessary for the physical decay of the isotope, provided that the standard was counted within a few minutes of the day t sample.

3) Cells were counted in a gamma counter (Gamma Set 500, I.C.N. Instruments, Belgium); at least 2500 counts were recorded in order to achieve an accuracy of 2%.
4.84. THE IN VIVO SURVIVAL OF DRUG-ENCAPSULATED ERTHROCYTES
AFTER STORAGE ON AGAROSE GELS, USING CHROMIUM-51 TO
LABEL THE CELLS.

It was decided that another gel system would be tested as a storage medium for erythrocytes. Agarose was used instead of gelatin and the same procedure was adopted as with the gelatin gels except that the cells were labelled with chromium-51 to monitor the in vivo survival.

1) Erythrocytes, which had been subjected to the encapsulation procedure (section 4.42) were brought to 37°C and incubated with various cytoplasmic nucleotides for 3 hrs. (See section 4.82.B. for details.)

2) 0.2g of agarose (Type I, Sigma Chemical Co, Poole) was made up to 40 ml in isotonic Hanks medium by gently heating the mixture until all the agarose had dissolved. The solution was aerated for 1 hr with 95% oxygen and 5% carbon-dioxide, followed by suspending the erythrocytes in the gel and set in the form of a "slope". The cells were then stored at 4xC for up to 14 days.

3) After prolonged storage, the gels were gently warmed and then centrifuged to remove the agarose. The recovered erythrocytes were then labelled with chromium-51 using the method described in section 4.83.

4) After reinfusion into the rat, regular tail vein samples were taken and thus the survival of labelled cells was determined.
4.85. THE EFFECT OF FREEZE-DRYING ON THE IN VIVO SURVIVAL OF DRUG-ENCAPSULATED ERYTHROCYTES.

The effect of freeze-drying the erythrocytes was investigated as a possible method of storing and transporting encapsulated preparations. To avoid the formation of ice-crystals during freezing, the erythrocytes were initially exposed to liquid nitrogen. The cells were then labelled with chromium-51 and returned to the rat's circulation to monitor their survival.

1) 0.1-0.2ml aliquots of drug-encapsulated erythrocytes were transferred into plastic tubes (Eppendorf tubes, Surrey) and immediately frozen in liquid nitrogen.

2) The frozen samples were then freeze-dried for 24 hrs in an automatic freeze-dryer (Viritis model 10-100).

3) After prolonged storage the cells were rejuvenated in a solution known as PIGPA (containing 9g NaCl, 50mmoles phosphate, 5mmoles adenine, 100mmoles glucose, 50mmoles pyruvate, adjusted to pH 7.2). 5ml of PIGPA was added to the red cells and incubated for 1 hr at 37°C in a shaking water bath.
4) The suspension was centrifuged and the supernatant discarded. The rejuvenated cells were then labelled with chromium-51 (section 4.83) and returned to the rat’s circulation via the femoral vein. Tail vein samples were taken at regular intervals and the survival of the labelled cells determined.
4.86. AN IN VITRO TEST INVOLVING DRUG-ENCAPSULATED ERYTHROCYTES BEFORE AND AFTER STORAGE.

OSMOTIC FRAGILITY TEST.

As a quick and simple method, the osmotic fragility test was very efficient, as a means of indicating the potential usefulness of an encapsulated preparation both before and after storage.

1) A range of sodium chloride solutions were prepared (0.1-0.9% in 0.02% increments).

2) 1 drop of the red cell suspension was added to 10ml of the saline solutions and left to settle for 30 mins. The tubes were remixed and centrifuged for 5 mins at 1200-1500g.

3) The colour of the supernatants was then measured spectrophotometrically (S.P.500, Pye Unicam, Cambridge) to give the percentage haemolysis, using the supernatant of the 0.9% NaCl tube as a blank.

4) The percent haemolysis produced by each concentration of sodium chloride was then plotted.
RESULTS.

5.1. THE SYNTHESIS OF CARBON-14 LABELLED LYS-PHE.

STAGE 1. FORMATION OF L-PHENYLALANINE BENZYL ESTER,
  P-TOLUENE SULPHONATE.

A creamy white solid was produced with a yield of 11.2g (80%). TLC of the final product gave a "blue" spot after spraying the plate with ninhydrin, therefore indicating a single product. The melting point was 170°C (theoretical value: 170-171°C), again indicating that the final product was very pure.

STAGE 2. FORMATION OF ALPHA, E-DICARBOBENZOXY-
  L-LYSINE.

A colourless oil was obtained after removal of the solvent with a final yield of 9.7g (78%). The purity of the product was determined by TLC and the plate was sprayed with iodine. Under exposure to ultra-violet illumination at 254nm, a single spot was visible, therefore showing the existence of a single product.
STAGE 3. FORMATION OF ALPHA,E-DICARBOBENZOXY-
L-LYSINE-L-PHENYLALANINE BENZYL ESTER.

After coupling the two protected amino acids, a white crystalline solid was formed with a final yield of 4.2g (67%). A single spot was detected under ultra-violet light at 254nm after TLC examination. The melting point of the product was 136°C (theoretical value: 139-142°C). Both tests proved that the product contained negligible impurities.

STAGE 4. FORMATION OF L-LYSYL-L-PHENYLALANINE DIHYDROCHLORIDE.

After addition of 1N HCl to the oil obtained from stage 3, a very pale yellow oil was formed. Difficulty arose in crystallizing the oil after the freeze-drying process and so it was decided that an equal amount of "cold" Lys-Phe would be added to aid the crystallization process. After vigorous stirring, the oil finally crystallized however the activity was reduced by a half due to this extra addition.
The final yield of the amorphous powder was 1.014g(72%) + 1.0114g("cold" Lys-Phe) = 2.028g.

The activity of the diluted product was measured and gave a value of 0.41mci.

TLC of the final product gave a single dark purple spot after spraying the plate with ninhydrin indicating the existence of a single product (i.e. the dipeptide).

The Rf value of the resulting product was found to be similar to those obtained from plates spotted with Lys-Phe purchased by Sigma Chemical Co, Poole, and with trituated Lys-Phe supplied by G.D. Searle & Co, High Wycombe.

### Rf VALUE

<table>
<thead>
<tr>
<th>Substance</th>
<th>Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon-14-LYS-PHE</td>
<td>0.46</td>
</tr>
<tr>
<td>LYS-PHE(Sigma)</td>
<td>0.47</td>
</tr>
<tr>
<td>Trituated LYS-PHE</td>
<td>0.47</td>
</tr>
</tbody>
</table>
5.2. THE IN VIVO SURVIVAL OF ERYTHROCYTES AFTER
ENCAPSULATION WITH LYS-PHE.

Fig. 5, shows the survival of cells after encapsulation with
Lys-Phe (Sigma) and saline ("sham" encapsulation). The values on
day 0 represent the percentage of FITC-labelled cells
introduced into the animals circulation i.e. the total blood
volume of the rat. It can be seen that the encapsulation
procedure did not affect the survival of the
cells, likewise, the presence of the dipeptide did not alter
the survival characteristics. The survival half-life value
for cells encapsulated with Lys-Phe was approximately 9-10
days.

Fig. 6, shows the survival of cells after encapsulation
with carbon-14, tritiated or Sigma Lys-Phe. The presence of
the radio-label on the dipeptide did not in any way affect
the survival of erythrocytes after encapsulation.

The effect of reinjection of erythrocytes into either
the same rat or into a different rat can be seen in Fig
7. Pooling the blood samples did not significantly produce
any changes in their survival patterns, however if
possible, encapsulated preparations were injected into the
same rat as from where the blood was obtained from.
FITC-labelled cells were visible in the circulation for up to 45 days, yet about 50% had disappeared within the first 10 days, indicating an initial rapid loss, followed by a slow steady decline.
FIG. 5. IN VIVO SURVIVAL OF FITC-LABELLED ERYTHROCYTES AFTER ENCAPSULATION.

☐ Represents the number of experiments.
FIG. 6. SURVIVAL CURVES FOR FITC-LABELLED ERYTHROCYTES
AFTER ENCAPSULATION WITH LYS-PHE.

○ Represents the number of experiments.
FIG. 7. SURVIVAL CURVES FOR FITC-LABELLED ERYTHROCYTES AFTER REINJECTION INTO THE SAME RATS, AND DIFFERENT RATS.

\( \bigcirc \) Represents the number of experiments.
% FITC CELLS

DAYS AFTER INJECTION

■■ same rat ⑥
■ different rat ⑥
5.3. The determination of counting efficiencies for tritium- and carbon-14 labelled Lys-Phe.

Table 1 shows that the chloroform/ethanol extraction method used for the removal of haemoglobin gave superior counting efficiencies for both tritiated- and carbon-14 labelled Lys-Phe as opposed to the values obtained from the digestion method. It was decided therefore, that the method of choice for the extraction of haemoglobin from erythrocytes during the assay of Lys-Phe would be the chloroform/ethanol extraction method.
<table>
<thead>
<tr>
<th>EXTRACTION METHOD</th>
<th>COUNTING EFFICIENCY.%(MEAN±S.D.)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CARBON-14 LABELLED</td>
<td>TRITIUM LABELLED</td>
<td></td>
</tr>
<tr>
<td>Chloroform/ethanol</td>
<td>82.88±1.95 (n=7)</td>
<td>16.27±2.11 (n=4)</td>
<td></td>
</tr>
<tr>
<td>Digestion</td>
<td>70.11±2.13 (n=7)</td>
<td>10.43±1.84 (n=4)</td>
<td></td>
</tr>
</tbody>
</table>
5.4. DETERMINATION OF THE LOCATION OF RADIOLABELLED LYS-PHE IN ERYTHROCYTES AFTER ENCAPSULATION.

Fig.8, shows the effect of increasing amounts of Lys-Phe on the percentage encapsulation. It can be seen that there is an optimum loading value for the drug added during the encapsulation procedure. Using a 40mM solution of carbon-14 labelled Lys-Phe, 400 micro-litres of this solution produced the maximum encapsulation. At lower levels, the amount of Lys-Phe encapsulated was markedly reduced. This suggests that a critical point must be reached, just prior to cell lysis, to enable the maximum amount of drug to be transported across the erythrocyte membrane.

With the addition of 400 micro-litres of Lys-Phe, 16.3% was found to be inside the cells (i.e. 860 micro-grams/ml packed cells). Under normal incubation conditions (i.e. incubating the cells with Lys-Phe for 2 hrs at 37°C), it was found that some of the drug had attached itself to the cell membrane and so this value was deducted to give the actual encapsulation.

Incubating 1ml packed cells with 400 micro-litres of Lys-Phe resulted in 2.3% of the drug being attached to the membrane, therefore the actual encapsulation value was 14.0% (740 micro-grams/ml packed cells). (Table 2.)
FIG. 8. THE INCORPORATION OF LABELLED LYS-PHE INTO RAT ERYTHROCYTES.

(Figures represent the number of determinations for each point.)
TABLE 2. THE EFFECT OF INCREASING AMOUNTS OF CARBON-14 LABELLED LYS-PHE ENCAPSULATED IN RAT ERYTHROCYTES.

<table>
<thead>
<tr>
<th>VOLUME OF LYS-PHE (micro-litres)</th>
<th>% TOTAL AMOUNT</th>
<th>% TOTAL AMOUNT</th>
<th>% (MEAN±S.D.) ACTUAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>3.0±1.1</td>
<td>1.2±0.3</td>
<td>1.8±0.6</td>
</tr>
<tr>
<td>150</td>
<td>3.0±0.9</td>
<td>1.5±0.3</td>
<td>1.5±0.3</td>
</tr>
<tr>
<td>200</td>
<td>3.6±1.7</td>
<td>1.8±0.6</td>
<td>2.8±0.1</td>
</tr>
<tr>
<td>300</td>
<td>8.0±1.9</td>
<td>1.7±0.8</td>
<td>6.3±0.8</td>
</tr>
<tr>
<td>400</td>
<td>16.3±2.7</td>
<td>2.3±1.1</td>
<td>14.0±1.1</td>
</tr>
<tr>
<td>450</td>
<td>12.2±1.1</td>
<td>2.1±0.3</td>
<td>10.1±0.7</td>
</tr>
<tr>
<td>500</td>
<td>cell lysis</td>
<td>1.9±0.5</td>
<td>-</td>
</tr>
</tbody>
</table>
5.5. THE RELEASE OF TRITUATED-LABELLED LYS-PHE FROM ERYTHROCYTES THROUGH DIALYSIS SACS.

Fig. 9, and Fig. 10, shows the release of methylene blue and labelled Lys-Phe respectively through dialysis sacs. From Fig 10 it can be clearly seen that labelled Lys-Phe encapsulated in erythrocytes diffused out through the dialysis sac at a much slower rate than either free Lys-Phe or non-encapsulated erythrocytes containing Lys-Phe. After 3 hrs, only 14% of the total trituated Lys-Phe added to the dialysis sac had leaked out, whereas 55% of the drug was found outside the sac containing non-encapsulated erythrocytes after the same time period.

The red cells themselves slowed down the leakage of the labelled drug, since in the absence of any erythrocytes, the dipeptide leaked out at a faster rate and over 80% had diffused out after 3 hrs, however cells that had been subjected to the encapsulation procedure inhibited diffusion more effectively.
FIG. 9. THE RELEASE RATE OF METHYLENE BLUE THROUGH A DIALYSIS SAC.
RATE OF RELEASE OF DYE (ABSORBANCE)

DIALYSIS TIME (HOURS)
FIG. 10. THE RELEASE RATE OF TRITIATED LYS-PHE AFTER ENCAPSULATION THROUGH A DIALYSIS SAC.

○ Represent the number of experiments.)
PAGES ARE MISSING IN ORIGINAL
PLATE 1. PHOTOMICROGRAPHS OF DEOXYGENATED SS-RBC.

TREATED WITH LYS-PHE

UNTREATED
PLATE 2. SCANNING ELECTRON MICROGRAPHS OF DEOXYGENATED SS-RBC.

TREATED WITH LYS-PHE

UNTREATED
5.8. ERYTHROCYTE ENTRAPMENT OF LYS-PHE BY AMPHOTERICIN-B WITHOUT HAEOMOLYSIS.

Fig. 11 shows that the optimum loading of labelled Lys-Phe/ml packed cells was achieved with a concentration of amphotericin-B = 7.5 micro-grams/ml. However, the percentage entrapped using this method was markedly lower than the value obtained from the encapsulation procedure using the same concentration of carbon-14 labelled Lys-Phe (8.9% as compared to 16.3% respectively).

The in vivo survival of FITC-labelled cells after entrapment was also shortened with a half-life of 5 days as compared to a value of 9-10 days for cells subjected to the encapsulation procedure (Fig. 12). Preincubated cells with amphotericin-B, but not containing Lys-Phe also had shorter survival times with a half-life of 5 days.

The rate of release of tritiated Lys-Phe from erythrocytes where the dipeptide was entrapped using amphotericin-B is shown in Fig. 13. After 3 hours, 48% of the total drug had diffused out into the surrounding medium, whereas only 14% of the labelled Lys-Phe had leaked out from cells that had been subjected to the encapsulation procedure over the same time period.

Table 4 shows that the dipeptide remained intact after the entrapment procedure, and also after leakage, thus indicating that the amphotericin-B did not in any way alter the structure of Lys-Phe.
FIG. 11. ENTRAPMENT OF LABELLED LYS-PHE BY AMPHOTERICIN-B 
WITHOUT HAEMOLYSIS.

Figures represent the number of determinations for each point.

1 ml of packed cells was incubated at 37\(^{\circ}\)C with different concentrations of amphotericin-B for 5 mins, followed by the addition of Lys-Phe (40 mM). After 30 mins., the cell suspension was washed thoroughly with isotonic phosphate-buffered saline containing EDTA, and the percentage of Lys-Phe entrapped was estimated.
FIG. 12. IN VIVO SURVIVAL OF CIRCULATING RAT ERYTHROCYTES
AFTER ENTRAPMENT OF LYS-PHE USING AMPHOTERICIN-B.

\( \bigcirc \) Represents the number of experiments.
FIG. 13. THE RELEASE RATE OF TRITIATED LYS-PHE AFTER ENTRAPMENT IN ERYTHROCYTES USING AMPHOTERICIN-B.

〇 Represents the number of experiments.
% $^3$H-lys-Phe released vs. dialysis time (hours)

- ■ free $^3$H-lys-Phe + amp-B (4)
- ▲ ▲ entrapped with amp-B (5)
- ● ● encapsulated (5)
TABLE 4. T.L.C. EXAMINATION OF ERYTHROCYTES ENTRAPPED WITH LYS-PHE USING AMPHOTERICIN-B.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Rf VALUE</th>
<th>NINHYDRIN SPRAY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon-14 labelled</td>
<td>0.46</td>
<td>DARK PURPLE SPOT</td>
</tr>
<tr>
<td>Lys-Phe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tritiated Lys-Phe (Searle)</td>
<td>0.47</td>
<td>DARK PURPLE SPOT</td>
</tr>
<tr>
<td>Entrapped carbon-14 labelled Lys-Phe</td>
<td>0.46</td>
<td>PURPLE SPOT</td>
</tr>
<tr>
<td>Dialysis sample containing entrapped Lys-Phe</td>
<td>0.47</td>
<td>FAINT PURPLE SPOT</td>
</tr>
</tbody>
</table>
6.1. THE RADIOIODINATION OF UROGASTRONE.

CALCULATION OF YIELD AND SPECIFIC ACTIVITY.

\[
\text{% IODINATION YIELD} = \frac{\text{COUNTS IN UROGASTRONE}}{\text{ORIGINAL COUNT}} \times 100
\]

COUNTS IN UROGASTRONE PEAK = ORIGINAL COUNTS - COUNTS IN 125-I IODIDE PEAK

\[
= 80.19 \times 10^6 - 46.91 \times 10^6
\]

\[
= 33.58 \times 10^6.
\]

THEREFORE,

\[
\text{% IODINATION YIELD} = 33.58 \times 10^6 \times 100
\]

\[
= \frac{80.1 \times 10^6}{80.1 \times 10^6}
\]

\[
= 41.9\%.
\]

RADIOACTIVITY INCORPORATED INTO UROGASTRONE =

\[
\text{%IODINATION YIELD} \times \text{ORIGINAL ACTIVITY} \times 100
\]

\[
= 41.9 \times 0.891
\]

\[
= 0.373 \text{ mci} = 373 \text{ micro-curies}
\]

THEREFORE,

SPECIFIC ACTIVITY = \frac{\text{UROGASTRONE RADIOACTIVITY}}{1.5}

\[
= \frac{373}{1.5}
\]

\[
= 249 \text{ micro-curies/micro-grams}.
\]
6.2. THE IN VIVO SURVIVAL OF CIRCULATING RAT ERYTHROCYTES AFTER ENCAPSULATION WITH UROGASTRONE.

Fig. 14, shows the in vivo survival of FITC-labelled erythrocytes after encapsulation with urogastrone. It can be seen that the overall lifespan of the erythrocytes was not shortened by the presence of urogastrone inside the cells.

A half-life of 9-10 days was observed for both encapsulated and control preparations.
FIG. 14. IN VIVO SURVIVAL OF CIRCULATING RAT ERYTHROCYTES AFTER ENCAPSULATION WITH UROGASTRONE.

○ Represents the number of experiments.
6.3. THE INCORPORATION OF IODINATED UROGASTRONE INTO RAT ERYTHROCYTES.

Using 125-I labelled urogastrone (10 micro-grams/ml), the optimum loading achieved was 17.6% i.e. 1.06 micro-grams/ml packed cells (Table 5 and Fig.15). It can also be seen that the encapsulation procedure resulted in a significant increase in the uptake of the labelled urogastrone as compared to simply incubating the cells with the peptide. Optimum encapsulation was reached as the cells were about to reach lysis, and this value decreased when lysis commenced.

The amount of labelled urogastrone that was attached to the cell membrane was measured at between 3-4%.
TABLE 5. THE EFFECT OF INCREASING AMOUNTS OF IODINATED UROGASTRONOE ENCAPSULATED IN RAT ERYTHROCYTES.

(MEAN ± S.D.)

<table>
<thead>
<tr>
<th>VOLUME OF 125-I (micro-litres)</th>
<th>% UROGAST.</th>
<th>% UROGAST. ATTACHED</th>
<th>% ACTUAL ENCAPS.</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>4.8±1.9</td>
<td>3.0±1.1</td>
<td>1.8±0.8</td>
</tr>
<tr>
<td>300</td>
<td>6.0±1.1</td>
<td>3.6±0.6</td>
<td>2.4±0.4</td>
</tr>
<tr>
<td>400</td>
<td>9.0±3.6</td>
<td>3.9±0.3</td>
<td>5.1±3.0</td>
</tr>
<tr>
<td>500</td>
<td>17.7±3.5</td>
<td>4.5±1.5</td>
<td>13.2±2.0</td>
</tr>
<tr>
<td>600</td>
<td>22.1±3.9</td>
<td>4.5±1.7</td>
<td>17.6±2.4</td>
</tr>
<tr>
<td>700</td>
<td>18.0±3.4</td>
<td>4.3±1.1</td>
<td>13.7±2.6</td>
</tr>
<tr>
<td>800</td>
<td>cell lysis</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
FIG. 15. THE INCORPORATION OF IODINATED UROGASTRONE INTO RAT ERYTHROCYTES.

(Figures represent the number of determinations for each point.)
6.4. THE RELEASE RATE OF IODINATED UROGASTRONE AFTER ENCAPSULATION THROUGH DIALYSIS SACS.

Fig. 16, shows the release of labelled urogastrone from erythrocytes, through dialysis sacs, over a 6 hr period. It can be seen that labelled urogastrone encapsulated in erythrocytes, diffused out through the dialysis sac at a much slower rate than either free urogastrone or non-encapsulating erythrocytes containing urogastrone. After 6 hrs, only 23% of the total urogastrone added to the dialysis sac had leaked out, whereas 60% of the peptide was found in the surrounding medium which had diffused out from sacs containing non-encapsulating red cells.

The red cells themselves slowed down the leakage of the labelled peptide, since in the absence of any erythrocytes, urogastrone diffused out at a faster rate and about 67% had leaked out after 6 hrs.

The results obtained from the examination of TLC plates (Table 6), show that the peptide remained intact after crossing into the cell, likewise, even after it had diffused out from the erythrocyte.
FIG. 16. THE RELEASE OF IODINATED UROGASTRONE FROM ERYTHROCYTES THROUGH A DIALYSIS SAC.

〇 Represents the number of experiments.
<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Rf VALUE</th>
<th>NINHYDRIN SPRAY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urogastrone (Sigma)</td>
<td>0.33</td>
<td>PURPLE SPOT</td>
</tr>
<tr>
<td>Iodinated urogastrone</td>
<td>0.32</td>
<td>PURPLE SPOT</td>
</tr>
<tr>
<td>Encapsulated urogastrone</td>
<td>0.32</td>
<td>LIGHT PURPLE SPOT</td>
</tr>
<tr>
<td>Dialysis sample containing urogastrone</td>
<td>0.32</td>
<td>FAINT PURPLE SPOT</td>
</tr>
</tbody>
</table>
6.5. THE INHIBITION OF CARBACHOL-INDUCED GASTRIC ACID SECRETION IN THE PERFUSED RAT STOMACH PREPARATION USING FREE AND ENCAPSULATED UROGASTRONE.

A typical trace is shown in Fig. 17, where after establishing a response with two increasing doses of carbachol, urogastrone preparations were added 5 mins prior to the addition of carbachol. The change in pH of the buffer was measured at its maximum. The fourth response acted as a control to bring back the carbachol-induced acid production; so as to repeat the experiment with an identical dose of urogastrone.

The responses expressed in terms of a "carbachol index" (C.I.), are shown in Fig. 18. A C.I. value of 0.43 was obtained using crude urogastrone (1 micro-gram/ml), whereas encapsulated urogastrone (approx. 1 micro-gram/ml packed cells) gave a value of 0.32. This indicated that free urogastrone was more effective in inhibiting carbachol-induced acid secretion than encapsulated urogastrone (Table 7).
The results of Fig. 19 show that even though encapsulated urogastrone did not inhibit the acid secretion as well as the free drug (54% inhibition as compared to 78% respectively), the duration of the effect was longer. 20% acid inhibition was still present after 2 hrs in the presence of encapsulated urogastrone whereas acid inhibition was completely abolished after 50 mins when free urogastrone was tested.

Cimetidine (40 mg) was found to be very effective in inhibiting acid production (87%), and the duration of its effect lasted for approximately 75 mins.
FIG. 17. INHIBITION OF CARBACHOL-INDUCED GASTRIC ACID
SECRETION THE PERFUSED RAT STOMACH PREPARATION.
RESPONSES (pH CHANGES) MEASUREMENT 45 MINS AFTER
INJECTION OF CARBACHOL(C), EXCEPT THIRD RESPONSE
WHICH WAS MEASURED AT MAXIMUM.
FIG. 18. RESPONSES IN TERMS OF CARBACHOL INDEX (C.I.) IN FIVE RATS GIVEN INCREASING DOSES OF UROGASTRONE: TWO SUCCESSIVE IDENTICAL DOSES (●, △) GIVEN TO EACH RAT.
TABLE 7. THE INHIBITION OF CARBACHOL INDUCED GASTRIC ACID SECRETION USING THE PERFUSED RAT STOMACH PREPARATION. RESPONSES EXPRESSED IN TERMS OF A CARBACHOL INDEX.

<table>
<thead>
<tr>
<th>GASTRIC ACID INHIBITOR</th>
<th>CARBACHOL INDEX (C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude urogastrone</td>
<td>Dose</td>
</tr>
<tr>
<td></td>
<td>(micro-grams)</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>Encaps. urogastrone</td>
<td>approx</td>
</tr>
<tr>
<td>1 micro-gram/ml cells</td>
<td></td>
</tr>
<tr>
<td>Sham encaps. (0.1% saline/ml packed cells)</td>
<td>0.0 (n=2)</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>40mg</td>
</tr>
</tbody>
</table>
FIG. 19. INHIBITION OF CARBACHOL-INDUCED GASTRIC ACID SECRETION IN THE PERFUSED RAT STOMACH PREPARATION AFTER INJECTION OF ENCAPSULATED UROGASTRONE.

○ Represents the number of experiments.
- cimetidine 40mg
- free urogastrone
- encaps. urogastrone
- sham encaps.
- control

% acid inhibition

% acid production

TIME / mins
6.6. **THE ANTI-SECRETORY ACTIVITY OF UROGASTRONE USING THE SHAY RAT MODEL.**

The results shown in Table 8 illustrate that encapsulated urogastrone was more effective in inhibiting the spontaneous acid secretion than free urogastrone. Both the volume of gastric juice and the acid output were reduced when compared to control experiments.

Cimetidine however, was more effective in reducing the volume and acid output compared to urogastrone preparations.
TABLE 8. THE ANTI-SECRETORY ACTIVITY OF UROGASTRONE USING THE SHAY RAT MODEL. (4 HOUR LIGATION).

(MEAN±S.D.)

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>VOLUME OF GASTRIC JUICE (ml/100g)</th>
<th>pH (meg/l)</th>
<th>ACIDITY (meg/100g)</th>
<th>ACID OUTPUT</th>
</tr>
</thead>
<tbody>
<tr>
<td>[NO. EXPTS.]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1ml saline</td>
<td>1.6±0.3</td>
<td>1.4±0.2</td>
<td>71±8.3</td>
<td>0.118±0.06</td>
</tr>
<tr>
<td>(0.9%) Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[4]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham encaps.</td>
<td>1.3±0.1</td>
<td>1.7±0.1</td>
<td>62±2.8</td>
<td>0.078±0.03</td>
</tr>
<tr>
<td>1ml packed cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[2]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free urogast.</td>
<td>0.7±0.2</td>
<td>2.1±0.1</td>
<td>59±3.0</td>
<td>0.051±0.05</td>
</tr>
<tr>
<td>(1 mcg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[2]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Encaps. urogast.</td>
<td>0.3±0.1</td>
<td>2.5±0.2</td>
<td>37±7.0</td>
<td>0.022±0.03</td>
</tr>
<tr>
<td>(aprox 1 mcg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[6]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cimetidine</td>
<td>0.2±0.1</td>
<td>2.3±0.1</td>
<td>42±3.1</td>
<td>0.027±0.07</td>
</tr>
<tr>
<td>(40mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[2]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
7.1. THE STORAGE OF DRUG-ENCAPSULATED ERYTHROCYTES ON GELATIN GELS.

From the initial experiments conducted to determine the nature of the gelatin gel to be used, it was decided that a 3% gelatin solution would be the most suitable strength (Table 9). These results also show that trypsin was not required to liquefy the gels since simply heating the gels liquefied them within minutes.

The effect of various incubation conditions of the erythrocytes on the storage and in vivo survival times can be seen in Table 10 and Fig. 20. Suspending the cells in gelatin increased, both the storage and in vivo survival times, likewise the addition of cytoplasmic constituents increased the mean survival time from 7 days, of cells lacking any nucleotide, to 20 days when incubated with adenosine, inosine, guanosine and glucose. Aeration of the gelatin solution prior to incubation, also significantly increased the survival time of the erythrocytes.

Fig. 21, shows that the half-life values for stored cells after reinjection into the rat were very poor to those obtained from fresh cells. Half-life values of 3-4 days were observed for cells stored on gelatin gels, compared to over 10 days for freshly prepared erythrocytes.
<table>
<thead>
<tr>
<th>Strength of Gelatin</th>
<th>Gelling Time (Hours)</th>
<th>Trypsin Added (mg)</th>
<th>Time to Liquefy (Secs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%</td>
<td>1</td>
<td>0.1</td>
<td>DID NOT GEL</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.5</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.1</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.5</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.1</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.5</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.1</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.5</td>
<td>40</td>
</tr>
<tr>
<td>2%</td>
<td>1</td>
<td>0.1</td>
<td>DID NOT GEL</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.5</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.1</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.5</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.1</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.5</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.1</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.1</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>3%</td>
<td>4%</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>----------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.1</td>
<td>DID NOT GEL</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>&quot;</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>265</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.1</td>
<td>142</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.1</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>413</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>149</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.1</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>97</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 10. THE EFFECT OF VARIOUS INCUBATION CONDITIONS OF DRUG-ENCAPSULATED RAT ERYTHROCYTES ON STORAGE AND IN VIVO SURVIVAL TIMES. (n=4 for all expts.)

<table>
<thead>
<tr>
<th>CONDITIONS</th>
<th>MEAN STORAGE TIME (DAYS ± S.D.)</th>
<th>MEAN SURVIVAL TIME (DAYS ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Encaps. Lys-Phe suspended in normal Hanks</td>
<td>4.5 ± 0.5</td>
<td>4.3 ± 0.8</td>
</tr>
<tr>
<td>B: Encaps. Lys-Phe suspended in 3% gelatin</td>
<td>1.8 ± 0.4</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>C: As for (B)+ equilibrated with Carbogen</td>
<td>6.0 ± 0.7</td>
<td>7.0 ± 0.7</td>
</tr>
<tr>
<td>D: As for (C)+ incubated with 20mM adenosine</td>
<td>6.3 ± 0.4</td>
<td>7.3 ± 0.8</td>
</tr>
<tr>
<td>E: As for (D)+ incubated with 5mM inosine</td>
<td>11.3 ± 0.4</td>
<td>10.3 ± 1.8</td>
</tr>
<tr>
<td>F: As for (E)+ incubated with 5mM glucose</td>
<td>14.8 ± 0.8</td>
<td>17.3 ± 2.3</td>
</tr>
<tr>
<td>G: As for (F)+ incubated with 25mM guanosine</td>
<td>16.0 ± 0.8</td>
<td>20.0 ± 1.4</td>
</tr>
<tr>
<td>H: As for (G), except [sham]encaps.</td>
<td>13.8 ± 0.8</td>
<td>18.3 ± 1.8</td>
</tr>
</tbody>
</table>
FIG. 20. THE EFFECT OF VARIOUS INCUBATION CONDITIONS (A-H, SEE TABLE 10 FOR DETAILS) ON ERYTHROCYTES ENCAPSULATED WITH LYS-PHE ON:

1. STORAGE TIMES, AND 2. IN VIVO SURVIVAL TIMES. (MEAN±S.D.).
FIG. 21. SURVIVAL OF CIRCULATING RAT ERYTHROCYTES BEFORE AND AFTER STORAGE ON GELATIN GELS.

○ Represents the number of experiments.
7.2. THE IN VIVO SURVIVAL OF DRUG-ENCAPSULATED ERYTHROCYTES AFTER STORAGE ON AGAROSE GELS.

Fig. 22, shows the effect of labelling the erythrocytes with chromium-51 on the in vivo survival times. The radiolabel did not alter either the overall lifespan of the erythrocytes or the half-life values.

Suspending the cells in agarose only slightly improved the mean survival time, and the half-life was also comparable to that of erythrocytes suspended in gelatin.

However, it can be clearly seen that suspending the cells in either type of gel drastically improves the storage times of erythrocytes. 5 days was the maximum storage time achieved for cells simply incubated with a variety of cytoplasmic constituents, whereas erythrocytes stored on the gel can be stored for up to 16 days.
FIG. 22. IN VIVO SURVIVAL OF CHROMIUM-51 LABELLED RAT ERYTHROCYTES BEFORE AND AFTER STORAGE ON AGAROSE GELS.

○ Represents the number of experiments.
% SURVIVAL

DAYS

-  10 -  20 -  30 -  40 -

-  20 -  40 -  60 -  80 -  100 -

-  51Cr-cells 4 -
-  encaps: fresh 4 -
-  stored on agarose / 14 days 4 -
### TABLE 11. MEAN SURVIVAL TIMES OF DRUG-ENCAPSULATED ERYTHROCYTES AFTER STORAGE ON GELATIN OR AGAROSE GELS \((n=4)\)

<table>
<thead>
<tr>
<th>CONDITIONS</th>
<th>STORAGE TIME (DAYS)</th>
<th>MEAN SURVIVAL (DAYS S.D.)</th>
<th>HALF-LIFE (DAYS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Encaps. Lys-Phe</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>suspended in normal Hanks</td>
<td>0</td>
<td>43.2±2.8</td>
<td>9-10</td>
</tr>
<tr>
<td>B: Encaps. Lys-Phe</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>suspended in normal Hanks</td>
<td>3</td>
<td>4.5±0.5</td>
<td>0-1</td>
</tr>
<tr>
<td>C: As for (A)+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>incubated with cytoplasmic const.*</td>
<td>5</td>
<td>12.0±1.4</td>
<td>2-3</td>
</tr>
<tr>
<td>D: As for (B)+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>suspended in 3% gelatin(aerated)</td>
<td>14</td>
<td>20.0±1.4</td>
<td>4-5</td>
</tr>
<tr>
<td>E: As for (C)+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>suspended in 0.5% agarose(aerated)</td>
<td>14</td>
<td>23.5±0.7</td>
<td>3-4</td>
</tr>
</tbody>
</table>

*cytoplasmic constituents: 20mM adenosine, 5mM inosine, 5mM glucose, 25mM guanosine
7.3. THE IN VIVO SURVIVAL OF DRUG-ENCAPSULATED ERYTHROCYTES AFTER FREEZE DRYING.

The method of freeze-drying the cells did not prove to be a suitable method for storing erythrocytes. Upon reconstituting the freeze-dried cells, a very thick suspension of lysed cells was formed which rendered the preparation useless.
7.4. AN IN VITRO TEST INVOLVING DRUG-ENCAPSULATED ERYTHROCYTES.

OSMOTIC FRAGILITY TEST.

Fig. 23, shows the osmotic fragility curves for red cell supernatants comparing erythrocytes subjected to either the encapsulation procedure or the procedure of entrapment using amphotericin-B. Lysis started at higher concentrations of NaCl for cells subjected to the entrapment procedure (0.7%), compared to cells from the encapsulation technique (0.55%), indicating that the former method rendered the cells more fragile.

For normal untreated cells, lysis started at 0.45% and was complete at 0.24%, whereas for cells subjected to either of the two loading techniques, lysis ended between 0.3-0.35% NaCl.

The dipeptide itself did not affect the osmotic fragility of the cells since an identical curve was obtained with the absence of Lys-Phe.

The effect on the osmotic fragility of erythrocytes after storage can be seen in Fig. 24. Cells stored on either gelatin or agarose began to lyse at greater concentrations of NaCl (0.75% and 0.85% respectively), and finished at concentrations of between 0.35 and 0.4%, confirming that storage did indeed make the cells more fragile.
FIG. 23. OSMOTIC FRAGILITY CURVES FOR RED CELL SUPERNATANTS

COMPARING DRUG-ENCAPSULATED AND DRUG-ENTRAPPED
ERYTHROCYTES.

☐ Represents the number of experiments.
FIG. 24. OSMOTIC FRAGILITY CURVES FOR FRESH AND STORED ERYTHROCYTES ENCAPSULATED WITH LYS-PHE.

[Diagram]

○ Represents the number of experiments.
DISCUSSION.

8.1. INTRODUCTION.

The project was concerned with an exploration of the therapeutic possibilities of drugs or other pharmacological active substances encapsulated in erythrocytes. Two specific projects were investigated: the use of the encapsulation technique to investigate the passage of Lys-Phe through the erythrocyte plasma membrane and the use of erythrocytes as slow-release vesicles using urogastrone as an example. Also investigated were general properties such as survival in vivo, loading characteristics and storage conditions, all these properties being related to the possible uses of erythrocytes in the clinic.

This method was used in all experiments as it was quick and simple in operation and also gave good loading and survival characteristics. The major disadvantage was that it was performed on a small scale and also the blood was exposed to the atmosphere hence the possibility of contamination and manipulation difficulties.
8.2. THE ENCAPSULATION OF EXOGENOUS AGENTS IN ERYTHROCYTES.

Erythrocytes have been proposed and recently utilized as carriers or vehicles for drugs, enzymes, pesticides, DNA molecules and other bioactive agents (DeLoach, 1983). For biologically active molecules, entrapment or encapsulation in erythrocytes may offer immunologic protection to the exogenous material, prevent premature degradation or inactivation, provides slow or controlled release into the circulatory system and target the compound to the reticuloendothelial system.

The loading procedure was carried out at room temperature, although other workers who have used this method employed lower temperatures. Pitt et al. (1983a), found difficulties when working at very low temperatures since the cells, when returned to the circulation, did not survive. The viscosity of the cell suspension was so increased as to cause problems in mixing. Working at ambient temperatures not only enabled the cells to survive in vivo but preserved the membrane charge at normal value.

The use of visual observation to judge the point of haemolysis was used for convenience. However, this method does not produce reproducibility from worker to worker.
As experience was gained during the encapsulation programme, the greater was the ease and reproducibility in determining the point of haemolysis. This point was judged to be when the colour of the packed cells and the supernatant was identical, and that the boundary between the cells and the supernatant appeared to be less distinct. Alternatively, the point of haemolysis can be determined experimentally by monitoring the osmotic pressure or determining the haematocrit value.

It was also observed during the course of the study that blood obtained from younger rats as opposed to older rats affected the point at which haemolysis occurred. The manner in which the blood was collected from the animal was also important; when the blood was collected very quickly, encapsulation was much easier and gave better loading values. These observations again emphasize the fact that as experience was gained, improved loading and reproducibility was achieved.

The method of entry of the drugs into the cells is unknown. Evidence presented by Seeman (1967), showed that the permeable state of the cells existed only between 15-25 seconds after the onset of haemolysis. Large particles such as ferritin or colloidal gold entered the cells which suggests that pores opened in the membrane. Electron micrographs of the transient holes fixed in the open position by glutaraldehyde in human erythrocytes have been published by DeLoach et al. (1980).
Trial encapsulations were carried out whenever a different drug or concentration was to be used. The concentrations of drugs used for encapsulation were calculated on the assumption that there would be equilibration of the drug between extracellular and intracellular compartments at the point of haemolysis. Ihler et al. (1973) reported that haemoglobin release and the entry of the enzyme beta-glucosidase were parallel events, and that haemoglobin concentration after haemolysis was found to be equal in the intra- and extracellular compartments of those cells which actually haemolyse.

Since not all the cells lyse at once, 100% equilibration cannot be expected in a normal heterogeneous population. The same workers found that 60% incorporation of extracellular material was found under optimal conditions.

A certain number of cells were destroyed or lost during the encapsulation process, and so the yield of the encapsulated preparation was smaller than the starting cell volume. Throughout this study a reduction from 1.0 ml to 0.7 ml was generally found. It would be reasonable to postulate that most of these cells were ones which had probably lysed and perhaps transiently encapsulated some of the drug.
This would account for the discrepancy between the amount of drug incorporated immediately after encapsulation and that found in the final preparation. Therefore, taking these factors into consideration, less than 50% encapsulation of a substance could be reasonably assumed.

In order to extend this method to the clinical situation, the procedure would have to be developed for large-scale operation. A standard plastic blood transfusion bag could be used as the working vessel to minimize handling and contamination. Recognition of the point of haemolysis would be crucial. At the very least, a standard colour card could be of assistance for matching with the appearance of the supernatant. Moreover, spectro-photometric techniques would give a better result quantitatively as well as qualitatively.
8.3. THE ENCAPSULATION OF LYS-PHE INTO INTACT ERYTHROCYTES.

8.31. THE IN VIVO SURVIVAL OF ERYTHROCYTES AFTER ENCAPSULATION WITH LYS-PHE.

The survival of the encapsulated preparation in the circulation was tested by intravenous administration to the rat. The labelling of the cells with FITC enabled re-transfused cells to be identified in the circulation for counting. The results indicated that the survival time of the cells was not altered by the encapsulation and labelling procedures. As experience was gained in the experimental and manipulative handling, there was an improvement in the percentage of cells reinjected into the rat's circulation.

The normal lifespan of rat erythrocytes is between 55 to 65 days depending on the method of determination (Bishop and Surgenor, 1964). From Fig. 4, it can be seen that both test and control cells were still present in the circulation 45 days after injection. There also appears to be little or no difference in the survival times of cells encapsulated either with Lys-Phe or "sham", indicating that the dipeptide was not in any way altering the characteristics of the red cell.
The decline of all labelled cell preparations seemed to follow an exponential decay curve; an initial rapid decay, followed by a less rapid decay. A 9-10 day half-life was observed for cells subjected to the encapsulation procedure, whereas a value of 11-12 days was seen for untreated cells. These results are consistent with those observed by other workers. Updike et al. (1976), encapsulated an anti-tumour agent, asparaginase, in monkey erythrocytes in order to avoid serious allergic reactions to the drug.

Encapsulation was achieved by hypotonic dilution and the resulting carrier erythrocytes circulated within the recipient monkey’s with a half-life of about 8 days. Alpar and Lewis (1985), using the same procedure for encapsulation as used for Lys-Phe reported half-lifes of 9-10 days in rats and 14 days in C3H mice when testing asparaginase. The electrical field procedures employed by Kinosita and Tsong (1978), yielded carrier erythrocytes capable of greater survival in vivo.
Part of the initial loss is probably associated with the removal and manipulation of the erythrocytes in vitro since such losses are observed with normal erythrocytes removed for chromium-51 labelling and then reinfused. Part of the erythrocyte population is also damaged by the lysis procedure. This damage was minimized by considering important parameters which include preservation of enzymes and cytoplasmic constituents and inclusion of substrates needed for ATP generation including glucose, adenosine and phosphate. Greater attention to the ion and small molecule content (e.g. glutathione) of resealed erythrocytes may help to diminish damage. Erythrocytes which survive the period of rapid loss, presumably those with the least damage and the most normal metabolism, nevertheless seem to be removed somewhat more rapidly than normal erythrocytes. Current research on erythrocyte aging has implicated a physiologic autoantibody in the removal of normal erythrocytes (Kay, 1978; 1981). Increased amounts of the antigen are found on cells as they age and it is possible that hypo-osmotic lysis might accelerate formation of this antigen.

Fig. 5, illustrates the effect of Lys-Phe, labelled with either carbon-14 or hydrogen-3, had on the in vivo survival of carrier cells. The survival characteristics were similar to cells loaded with Lys-Phe supplied by Sigma proving that the radiolabel was not in any way disrupting the red cell.
From the results shown in Fig.6, it can be noticed that the initial decay of FITC-labelled erythrocytes from pooled blood was slightly faster than that seen from erythrocytes injected back into the same rat. This subsequently meant, that if possible, re-injections were carried out in the same rat since any factor which may cause an increased uptake and destruction of labelled cells by the reticuloendothelial system would decrease the efficiency of the erythrocyte as a slow-release drug carrying mechanism.

The labelling of erythrocytes with FITC can be used in two ways. One method is by labelling the drug to be encapsulated (Pitt et al., 1983a), or alternatively, is by labelling the erythrocytes. The latter method was used throughout this work.

After incubation with FITC, the cells appeared very brilliant in ultra-violet after preparation and immediately after transfusion. After 24 hours, the fluorescence diminished but the cells were still easily distinguishable from unlabelled cells. Under tungsten light, there was no difference in the appearance of labelled and untreated normal cells.
From these initial experiments, it can be concluded that this method of encapsulation gave very good survival times of the cells in vivo, when compared with results obtained by other workers. For example, Tyrrell and Ryman (1976), used a hypotonic dilution method to entrap methotrexate. When rats were injected with labelled carrier cells, over 90% of the radioactivity was found in the liver and spleen at 1 hr. postinjection. Thus, the circulating survival of these cells exhibited a half-life of about 30 mins. Ang et al. (1977), studied the encapsulation parameters of beta-galactosidase in chicken erythrocytes by hypotonic dilution; these cells survived in the circulation with a 5-day half-life, which is about half that of normal erythrocytes.
8.32. THE SYNTHESIS OF CARBON-14 LABELLED LYS-PHE.

Difficulty arose in the crystalization of the oil during stage 4 of the reaction, however, when trituated with an equal amount of "cold" Lys-Phe, a dull white crystalline solid was formed. Obviously, due to this dilution, the activity was reduced but the final activity of 0.41 mci proved to be sufficient for subsequent experiments. The tritium-labelled dipeptide supplied by G.D. Searle and Co. Ltd, had a higher activity but because of its low counting efficiency, the results obtained during the assay were very variable.

TLC experiments and ultra-violet scans of the labelled dipeptides confirmed that the products were pure and that a total racemisation of the product occurred.
8.34 THE DETERMINATION OF COUNTING EFFICIENCIES FOR TRITIUM-AND CARBON-14 LABELLED LYS-PHE.

Counting efficiencies were obtained using the internal standardization technique, and produced very consistent results. The advantage of this technique is that it can be applicable to all types of quench in the sample. Correction by a quench curve is not necessary in this method. This method is also very rapid for small numbers of samples. There are a number of disadvantages that make the internal standardization method less popular than other methods; there is always the problem in reopening the sample in terms of time and convenience. Secondly the sample cannot be recounted once the spike has been added. The method is subject to pipetting errors in the introduction of the spike. The spike itself may quench the sample further and lead to low counting efficiencies.

The assumption in this method is that the spike behaves exactly as the sample in terms of susceptibility to quench and physical intimacy in the cocktail; namely that the efficiency of counting the spike must equal the efficiency of counting the sample \( \text{Eff(sample)} = \text{Eff(spike)} \). The spike must be identical to the samples in terms of chemical character. If the sample is aqueous, for example, erroneously high counting efficiencies will be obtained by using an organic standard such as radioactive toluene.
The disadvantage incurred when using the digestion method to prepare the cells for counting was chemiluminescence which resulted in high background rates which took several hours to decay. Chemiluminescence is often generated during sample preparation when strong bases are used to digest biological samples, however, neutralization with hydrochloric acid proved to be effective.

The problem of chemiluminescence did not pose to be a nuisance when using the chloroform/ethanol extraction method. After centrifugation, a clear supernatant was obtained and counting was possible after 20 mins. The digestion method also resulted in the production of a pale-yellow tinge, after the addition of hydrogen peroxide, resulting in variable counting rates. With larger blood samples a very distinct yellow, hazy solution was formed. It was decided to use the chloroform/ethanol extraction method for all future experiments.

Carbon-14 labelled Lys-Phe gave very good counting efficiencies (83%), compared to the tritiated form (16%). Therefore, the former type was used for the assay of the dipeptide after encapsulation since counting errors would be significantly reduced due to the high counting efficiency.
8.35. THE INCORPORATION AND LOCATION OF RADIONLABELLED LYS-PHE INTO ERYTHROCYTES.

The results show that the encapsulation procedure resulted in a significant increase in uptake of labelled Lys-Phe. Under optimal conditions, 16% of the dipeptide had entered the cells as opposed to about 2% in cells simply incubated for 2 hrs. This value is lower than the theoretical maximum. At the point of haemolysis the extracellular and intracellular volumes are approximately equal, and if the substance is distributed equally, then 50% would be within the cells. DeLoach and Ihler (1977), reported that ghosts prepared by hypotonic dialysis can take up between 30-60% of added drug; however, it is doubtful that using this system such figures could be obtained. Ihler et al. (1973), using hypotonic dilution as a method of encapsulation expected a value of between 1 and 8%, and therefore the value of about 14% for the dipeptide using the minimum damage method is very favourable.

Pitt et al. (1983a), encapsulated a range of drugs and obtained values of between 6-20% using the same system. The same workers also showed that using a conjugate of FITC and methotrexate, at least 20% of the cells remained empty at the point of lysis.
The results also show that hardly any of the drug was taken up by the cells until the point of lysis was reached. At this point there was a sudden and drastic influx of the dipeptide up to to a maximum, followed by a slight decrease before the cells lysed. This seems to indicate that the pores in the membrane are only open at this point when the cells are capable of taking in the maximum amount of drug. These results support the findings of Seeman (1967) where he was able to demonstrate the existence of transient "holes", of the order of 200 to 500 Angstroms wide, in the erythrocyte membrane. He was able to fix the open holes by adding glutaraldehyde 10-20 secs. after the onset of haemolysis. This would therefore seem to indicate that the "holes" are only fully open at the point of lysis and hence the maximum amount of drug can enter the cells.
8.36. **THE RELEASE OF LABELLED LYS-PHE FROM ERYTHROCYTES THROUGH DIALYSIS SACS.**

Most drugs tend to exit from erythrocytes much too quickly to achieve a sustained release and the rate of exit is proportional to the instantaneous intracellular drug concentration (first order kinetics), instead of being constant with time (zero order kinetics). Erythrocyte carriers do however have the potential of zero order kinetics which is of great significance to drug delivery. Erythrocytes are not selected at random for destruction. Instead they have a specific potential life time and undergo a poorly characterized aging process. New cells are manufactured at a rate that is fairly constant, but which can be accelerated if there is an acute need for more erythrocytes. Old cells are ordinarily destroyed at the same rate as new cells are made.

The use of dialysis sacs was employed as an analogue of the erythrocyte membrane to investigate if slow leakage through the cell was a likely mode of action for the release of encapsulated material.

The experiments show that the free dipeptide crossed the dialysis membrane at a rate proportional to the concentration difference between the inside and outside of the membrane; whereas the erythrocyte-encapsulated preparation inside the dialysis sac, released Lys-Phe at a fairly constant rate.
It can be seen that the presence of the erythrocytes themselves was not responsible for the slow leakage, but the fact that the drug was actually encapsulated inside the cell caused it to be released very slowly.

From the results it seems that Lys-Phe was released from encapsulation preparations at a constant rate/hr, however, more detailed pharmakokinetics profiles would need to be determined before claiming that this system was acting as a "true" slow-release mechanism.

Confirmation that Lys-Phe remained intact when either entering the cell or after leakage from the erythrocyte was determined by TLC experiments. In both cases the existence of a single purple spot on the plate indicated that the dipeptide was not broken down.
8.37. ERYTHROCYTE ENTRAPMENT OF LYS-PHE BY AMPHOTERICIN-B WITHOUT HAEMOLYSIS.

Amphotericin-B is a polyene antibiotic that binds to sterols and perforates cell membranes. Kitao and Hattori (1980), have used this agent to develop a unique method for drug encapsulation. Amphotericin-B premixed with erythrocytes facilitated the entrapment of the antileukaemic drug, daunomycin, in both human and mouse erythrocytes. This method of entrapment yielded carrier cells with unaltered haematological parameters.

Lys-Phe was entrapped using this method, and the results show that the maximum percentage entrapment achieved was approximately 9%. This value was considerably lower than that obtained by the encapsulation procedure. The amount of amphotericin-B added to the cells was critical; 7.5 mcg/ml producing the optimal entrapment. Kitao and Hattori (1980), found that a concentration of 6 mcg/ml produced the maximum entrapment of daunomycin. At higher concentrations the cells were found to lyse and therefore it would seem likely that low concentrations of amphotericin-B caused transient membrane lesions that enhance the uptake of Lys-Phe.

This process was also reversible and can be controlled because the increased transport stopped when the cells were washed free of amphotericin-B. A short exposure time to amphotericin-B was found to be non-toxic to the cells (Kumar et al., 1974).
The survival times of resealed cells in vivo were very poor. A half-life of 5 days was observed, compared to between 9-10 days for cells subjected to the encapsulation procedure. Therefore it seems that in causing the transient lesions in the cell membrane, the amphotericin-B was also disrupting the membrane to such an extent that the haematological parameters were altered.

Efflux of Lys-Phe from entrapped erythrocytes in vitro revealed that 48% of the drug was released in 3 hrs., compared to only 14% from encapsulated preparations. Therefore for this particular drug, the method of entrapment using amphotericin-B was inferior with respect to the cells' ability to retain the dipeptide. This however, does not mean that the method is less superior for the entrapment of other drugs, and more detailed studies employing a variety of drugs of differing molecular size and shape would need to be investigated.

Drugs could easily be encapsulated in a form that is not released from the erythrocyte at any significant rate, for example by incorporating them linked to a polymer. The drug however, could be released from macrophages after erythrophagocytosis if the linkage is susceptible to lysosomal but not erythrocyte enzymes. If the drug is encapsulated in a random population of erythrocytes, then a constant fraction of the cells will be removed each day and a constant amount of drug will be made available each day.
8.38. THE ENCAPSULATION OF LYS-PHE INTO INTACT SS-RBC.

Attempts to design a non-covalent and non-toxic anti-sickling agent have included the examination of oligopeptides and amino acids (Noguchi and Schechter, 1977). Derivatives of phenylalanine were more effective inhibitors of the polymerization of deoxy-HbS, and this inhibition has been correlated with the effects of varying charge and hydrophobicity in phenylalanine related compounds (Gorecki et al., 1980a). A series of small molecules were designed to maximize hydrophobicity and red cell membrane permeability and these included the benzyl esters of aromatic amino acids (Gorecki et al., 1980b). No major effects on the properties of deoxy-HbS solutions were observed, and it was concluded that these compounds acted on the membrane of the sickle erythrocyte to alter its properties.

Franklin et al. (1983), described how the dipeptide Lys-Phe combined the property of raising the solubility of HbS to that found in asymptomatic heterozygotes, with the ability to prevent sickling of erythrocytes.
From the photomicrographs it can be seen that Lys-Phe, encapsulated into intact SS-erythrocytes, inhibited the sickling of erythrocytes. The effect of Lys-Phe on the membrane of the sickle erythrocyte was probably a consequence of its combined charge and hydrophobicity; the lysine moiety favouring binding to the clusters of negative charges on the cell surface, while the phenylalanine conferred membrane solubility. How this binding inhibits sickling is unclear; it could be that the effect of the Lys-Phe is on the membrane-associated haemoglobin which may act as a nucleation centre for polymerization (Allan et al., 1982). Any membrane effect is not the same as that of L-phenylalanine benzyl ester, which causes cell swelling, perhaps by interfering with the sodium-ATP pump.

It is interesting to note that inhibition of sickling occurred more rapidly than the rate of movement of the compound into the red cell; hence suggesting a membrane-mediated effect.
Permeability studies with carbon-14 labelled peptides have showed that these compounds do not enter the intact erythrocyte under usual conditions of incubation (Votano et al., 1977), and therefore encapsulation could be a possible solution to overcome this problem. Kumpati (1982), employed a liposomal transport system to transfer phenylalanine into intact SS-erythrocytes, and demonstrated a marked inhibition in the in vitro sickling of deoxy-HbS. These findings, together with the results obtained from the encapsulation of Lys-Phe may have significant therapeutic implications in the treatment of sickle cell disease.
8.4. THE ENCAPSULATION OF UROGASTRONE INTO INTACT ERYTHROCYTES.

8.41. THE RADIOIODINATION OF UROGASTRONE.

There are many methods available for the labelling of proteins with iodine-125, such as electrolytic iodination, enzymatic iodination, iodine monochloride method and the chloramine-T method. The latter method was used for the iodination of urogastrone.

The chloramine-T reaction is technically simple and rapid to perform and is thus the method of choice in the first instance when setting out to iodinate a protein for the first time. High incorporation of radioiodine into proteins is generally obtained. Some proteins appear to be readily damaged by this method particularly by the formation of aggregated material, and the enzymic activity of some proteins seems to be lost after exposure to oxidizing agents (Sherman et al., 1974).

In the case of urogastrone, a tracer solution with a specific activity of 249 micro-curies/mcg was prepared, with an iodination yield of about 42%. It was important that the minimum concentration of oxidizing agents needed was employed. Higher concentrations are a potential cause of decomposition of the peptide.
The reaction appeared to be instantaneous and minimum exposure of the urogastrone to the harmful reagents was achieved by the addition of the reagents as rapidly as possible, followed immediately by the purification of the labelled urogastrone.

The chloramine-T reaction appears to have a pH optimum of around 7.5, the yield being reduced below 6.5 and above pH 8.5. 125-iodide obtained commercially was in NaOH at pH 8-10 and this therefore had to be adjusted by the addition of a buffer at pH 7.5 before use. Above pH 8.0-8.5 the substitution of iodine into the imidazole ring of histidine is favoured (Ganguli and Hunter, 1971).

The biological activity of the labelled urogastrone was subsequently tested experimentally on the perfused rat stomach.
8.42. THE IN VIVO SURVIVAL OF CIRCULATING RAT ERYTHROCYTES AFTER ENCAPSULATION WITH UROGASTRONE.

Urogastrone has been isolated in yields of about 1mcg/l human urine and the structure shown to consist of a single polypeptide chain of 53 amino acid residues with three internal disulphide bonds (Gregory and Preston, 1977). A second molecular species was isolated that lacked the C-terminal arginine residue. Urogastrone was shown to be a powerful and specific inhibitor of gastric acid secretion against any stimulant used in a number of different species (Hollenberg, 1979).

Initially urogastrone was encapsulated into erythrocytes at a concentration of 10mcg/ml packed cells. Survival curves were produced and it can be seen that urogastrone did not alter the haematological parameters of the carrier erythrocytes. Urogastrone containing a tracer solution of 125-I-urogastrone also exhibited a similar survival curve for resealed erythrocytes, with a life-span of about 45 days and a half-life in the order of between 9-10 days. This demonstrated that the tracer solution was in fact homogenous and that the 125-iodine was not in any way disrupting the erythrocyte.
8.43. THE INCORPORATION OF LABELLED UROGASTRONE INTO INTACT ERYTHROCYTES.

As with the encapsulation of Lys-Phe, there was an optimum value for the loading of urogastrone into erythrocytes. Up to the point of haemolysis, the uptake of urogastrone was very slow, but at the point of lysis, uptake was significantly increased. This further supports the theory that membrane lesions are open only for a very short time and it is only then that the drug is taken up in any noticeable quantities.

Incubating the erythrocytes with equivalent amounts of urogastrone resulted in about 3-4% of the drug to be attached to the cell membrane and therefore it can be concluded that the uptake of the peptide is greatly increased by encapsulation.
8.44. THE RELEASE OF LABELLED UROGASTRONE AFTER ENCAPSULATION IN ERYTHROCYTES THROUGH DIALYSIS SACS.

The results show that urogastrone was released at a slower rate from encapsulated preparations. Once again, it can be clearly seen that it was the encapsulation procedure itself, and not the mere presence of the erythrocytes that caused the drug to leak out at a slower rate. It is interesting to note that urogastrone leaked out at a faster rate than Lys-Phe over the same time period. It would be expected that urogastrone, being a larger molecule, would diffuse out from the cell at a slower rate than the dipeptide. Thus, it would seem that molecular size alone is not the rate determining step, but probably a combination of various factors such as polarity, structural alignment and molecular size.

Murray et al. (1983), have developed a technique for ensuring the controlled release of microgram and smaller amounts of biologically active epidermal growth factor (EGF) from polymeric delivery systems. Albumin in milligram quantities facilitated the sustained release of picogram amounts of EGF for at least 3 weeks. The EGF-containing polymer matrix was placed directly into cell culture and was found to increase the proliferation rate of serum-starved cells.
From the results of the in vitro release of urogastrone from erythrocytes, it can be calculated that approximately 0.25 mcg urogastrone had diffused out from the cells over a 3 hr. period which indicates that urogastrone, encapsulated into erythrocytes, has the potential of a controlled delivery system.

TLC experiments demonstrated that urogastrone was not broken down when after entering, or after leaking out from the cell and therefore should remain biologically active after encapsulation into erythrocytes.
8.45. THE ASSAY OF ENCAPSULATED UROGASTRONE USING THE PERFUSED RAT STOMACH PREPARATION.

Ghosh (1958) showed that urogastrone produced a dose-dependant inhibition of histamine-induced gastric acid secretion using the perfused rat stomach preparation. He found the effects long-lasting which presented problems in quantitating results. Roesenoer and Schild (1962) developed the earlier work on short-acting inhibitors to enable four doses of urogastrone to be given to one rat. Carbachol was found to be a more reliable stimulant for inhibitory assays than histamine although the side effects sometimes created problems. Analysis of variance showed no significant deviations from parallelism between standard and test nor a significant difference between rats. The procedure was not entirely satisfactory for routine use since only 25% of the animals could be given four doses of inhibitor.

Lawrence et al. (1971), used a simpler assay system in which only two doses of inhibitor were given to each animal. The inhibitory effects of urogastrone were expressed in terms of the dose-response curve of carbachol which gave more consistent results than expressing it simply in terms of pH changes.
A test of deviation from parallelism could not be applied but this was not a serious problem when test and standard originated from the same source. A succinic-propionic buffer was used which gave a near-linear change in pH when hydrogen ions were secreted. The buffer was continuously recirculated through the rat's stomach so that hydrogen ions accumulated and the response was integrated as it occurred. A simple linear measurement gave the amount of acid secreted in a given time and the time course of secretion could be continuously followed by a glass electrode.

In order to obtain a steady baseline and reproducible results it was essential to have a clean stomach with no obstruction to the outflow through the pyloric cannula. The length of the cannula was designed to fill the body of the stomach and the perforations allowed free flow of the buffer.

The first response observed in the anaesthetized rat was found to be unreliable and therefore it was rejected in the assay. The variability with successive doses was usually small with no systematic trend in responsiveness.
The use of an animal unit was found to be of practical convenience. Urogastrone activity was measured by reference to the log dose-response curve of carbachol by means of a "carbachol index", and a unit of activity derived from this index has been adopted (Lawrence et al., 1971). The unit is defined as the amount of urogastrone which, when injected intravenously into an anaesthetized rat, produced a carbachol index of 0.15.

The results show that free urogastrone was more effective in inhibiting gastric acid secretion than encapsulated preparations, however the duration of action was not as long in the former case. After 60 mins, the effects of the free inhibitor were completely abolished and the basal acid production had commenced.

With encapsulated urogastrone, inhibition of acid secretion was still observed after 2 hrs. These results can be correlated with the findings from the in vitro release experiments where urogastrone was still present in the erythrocytes after 3 hrs. These results seem to indicate that encapsulated urogastrone could have the potential as a slow-release system in the inhibition of gastric acid secretion.
This technique of assay has also been used for other pharmacological studies. Colin-Jones and Himsworth (1969), used it to investigate the characteristics determining the gastric acid response to hypoglycaemia. More recently radioimmunoassay (RIA) techniques have been developed in assaying urogastrone. Iodinated urogastrone was used to develop an RIA capable of measuring 5 pg peptide, and volumes of 1 to 2 ml human urine were thus assayed (Gregory et al., 1977).

Within a few minutes of an intravenous injection of urogastrone, the blood level became undetectable and the half-life was of the order of 2 mins. This is much shorter than the half-life of peptides of comparable size such as insulin or the smaller 35-residue big gastrin. The biological effect was sustained for a much longer period in that complete inhibition of the histamine-induced secretion was obtained at 15 to 30 minutes and only began again 75 mins post-injection, even though the secretagogue was infused continuously. Given subcutaneously, urogastrone was measured in the blood for a much longer period and the inhibitory effect was greatly prolonged even though the onset was still rapid (Gregory et al., 1979).
The dispersion of iodinated urogastrone in mice followed by whole body autoradiography has shown an accumulation of radioactivity in the gastric mucosal region with a gradual labelling of the stomach contents. However, this was subsequently found to be low molecular weight material that may have derived from degradation at some site of action in the mucosa. It was also observed that radioactivity appeared rapidly in the bladder (Gregory, 1980). The collection of urine by catheterization of female dogs confirmed that urogastrone appeared in the urine from 2 to 5 mins. after intravenous doses (Elder et al., 1978). The concentration in human urine is much higher than the total immunoreactivity in serum, which does not apply with hormones such as chorionic gonadotrophin and suggests that rapid clearance from serum is an important facet of this peptide.
8.46. THE ANTI-SECRETORY ACTIVITY OF UROGASTRONE USING

THE SHAY RAT MODEL.

The pylorus-ligated rat preparation, first described by Shay et al. (1945), involved the ligation of the pylorus under anaesthesia. At a predetermined time the animal was killed, the stomach removed and the acidity of the gastric juice measured. This method however, lacks specificity since the assays are performed against more or less spontaneous rather than drug-induced secretion. The advantages of this preparation lie in its simplicity and the absence of complicated or expensive apparatus, and once the conditions have been carefully controlled, the results are reliable. The principle disadvantage of the Shay rat is that large numbers of rats are required per group with the result that the procedure is time-consuming. Since only one test can be performed in each animal, the error will be increased due to large "between-animal" variations.

The results show that encapsulated urogastrone was comparable to cimetidine with respect to the anti-secretory action, since both the volume and acidity of gastric juice was reduced.

At this point, however, it must be mentioned that due to experimental difficulties, the sample population was too low to make any meaningful conclusions. To satisfy legal conditions in this country, animals were not allowed to recover from anaesthesia; maintaining anaesthesia for up to 4 hrs. proved to be very difficult and more than often the animal died before the completion of the ligation period.
8.5. POSSIBLE STORAGE SYSTEMS FOR DRUG-ENCAPSULATED ERYTHROCYTES.

8.5.1. THE STORAGE OF DRUG-ENCAPSULATED ERYTHROCYTES ON GELATIN AND AGAROSE GELS.

Although the mechanism with which erythrocytes maintain their biconcave shape during storage has been studied by a number of investigators, it has not yet been clarified. Nakao et al. (1959) reported that the 2,3-diphosphoglycerate and ATP contents of preserved blood, which are considerably decreased during storage, are restored to some extent by incubation with inosine or adenosine and that the ability of these cells to survive in the circulation after transfusion is also improved. After storage for much longer periods, however, the ATP level is not increased any longer by addition of inosine alone.

Hubbard et al. (1979), found ATP concentrations 63% of normal in mouse erythrocytes that were prepared by the dialysis procedure (Sprandel et al., 1979), employing buffers containing glucose and inosine.
These workers also reported that if buffers contained glucose, adenosine, phosphate buffer and 4mM magnesium chloride, ATP concentrations were restored to normal levels. Kostic et al. (1981), further recognized the importance of adenosine on the metabolism of stored and fresh human cells. Incubating the cells with adenosine resulted in a strong protective effect on 2,3-diphosphoglycerate.

The results show that incubating drug-encapsulated erythrocytes with various nucleotides increased the survival and storage times, and these times were further improved by the incorporation of gelatin or agarose prior to storage. Under optimum conditions, cells suspended in an aerated gel after incubation with various cytoplasmic constituents were able to be stored for up to 10 days, and when returned to the circulation, they survived for nearly 3 weeks. The fact that gelatin or agarose was used hardly affected the results. It was observed during these experiments that when the gels darkened upon storage, the cells were not able to survive in vivo. This probably indicated that the haemoglobin was being reduced to methaemoglobin and thus proved a valuable visual test for different preparations.
Schmer et al. (1979), fixed enzyme-containing erythrocyte ghosts to a gel and suggested that this system might have some potential for extracorporeal blood circulation. They showed that Km values for urease and glutaminase were essentially unchanged, and that there was no loss of enzyme activity after 8 weeks of storage or after extracorporeal circulation of the blood of a sheep for 30 mins. 

Even though mean survival times were greatly improved using this method of storage, the half-life observed of cells after reinjection was only in the order of 3-4 days, indicating that a large proportion of cells were damaged or have lost their shape during storage and are immediately taken up by the reticuloendothelial system. Hubbard et al. (1979), also reported that mouse erythrocytes prepared with glucose and inosine displayed a two-component survival curve, with half the cells being removed within 4 hrs. and more than 7 days elapsing before the next half was removed.

In rabbits on the other hand, most of the erythrocytes were rapidly removed within a few hours, although the survivors of this early loss displayed a half-life of about 4 days. In the dog, no early loss of erythrocytes was observed and the resealed erythrocytes had a normal half-life of 18 days (Sprandel et al., 1980).
Many blood banks use citrate- phosphate- dextrose (CPD) as the anticoagulant- preservative solution of choice. However recently, solutions containing citrate-phosphate- dextrose- adenine- one (CPD-1) and (CPD-2), where the adenine and glucose content was increased have improved the viability of red blood cells following extended storage (Sohmer et al., 1982).

Clearly, it can be seen that there are many methods of storing red cells and the use of gels is obviously limited as seen by the poor storage times. Careful modification of the incubation solutions and/or altering the nature of the gel could overcome this problem, and therefore potentially be a superior system.
8.52. THE FREEZE-PRESERVATION OF DRUG-ENCAPSULATED ERYTHROCYTES.

The method of freeze-preservation of red cells has been extensively researched and is probably the method of choice for storage. However, from the results of the experiments conducted, it can be seen that simply freeze-drying the cells, render them useless. The cells were first exposed to liquid nitrogen in order to avoid the formation of water crystals, followed by freeze-drying for 24 hrs. Upon rejuvenation, a viscous suspension of cells resulted.

Valeri (1975), developed a method where glycerol was added during the freeze-preservation process. Erythrocytes were freeze-preserved with either 20% w/v glycerol at -150 °C using liquid nitrogen, or with 40% w/v glycerol at -80°C using mechanical refrigeration. The washed red blood cells were stored in a sodium chloride-glucose-phosphate solution for at least 3 days, and after transfusion were therapeutically effective.

The osmotic fragility test has been shown to be a sensitive model for assessing red cell stability in vitro (Valeri, 1965). In carrying out osmotic fragility tests three variables capable of markedly affecting the results must be controlled, quite apart from the accuracy with which the saline solutions have been made up. Firstly, the relative volumes of blood and saline; a proportion of 1 volume of blood to 100 or 200 volumes of saline is convenient because the concentration of blood is so small that the added plasma hardly affects the osmotic effect of the saline.

Secondly, the final pH of the blood in the saline suspension must be controlled and finally, for really accurate work, the estimations should always be carried out at the same temperature, though for most purposes room temperature is sufficiently constant.

The osmotic fragility of freshly taken red cells reflects their ability to take up water without lysis. This is determined by their volume to surface area ratio.
The ability of the normal red cell to withstand hypotonicity results from its biconcave shape which allows the cell to increase its volume by about 70% before the surface membrane is stretched: once this limit is reached lysis occurs (Valeri 1965).

Spherocytes have an increased volume to surface area; their ability to take in water before stretching the surface membrane is thus more limited than normal and they are therefore particularly susceptible to osmotic lysis. The increase in osmotic fragility is a property of the spheroidal shape of the cell and is independent of the spherocytosis.

The results show that cells subjected to the encapsulation procedure, and moreover the entrapment procedure using amphotericin-B, have an increased osmotic fragility i.e. lysis began at higher concentrations of saline.

The osmotic fragility of stored cells on either gelatin or agarose was also increased. These increases were probably caused by swelling of the cells associated with an accumulation of sodium which exceeds loss of potassium. Such cation exchange is determined by the membrane properties of the red cell which control the passive flux of ions and the metabolic competence of the cell which determines the active pumping of cations against concentration gradients.
During incubation the metabolism of the red cell becomes stressed and the pumping mechanisms tend to fail, one factor being a relative lack of glucose in the medium.

These results are consistent with the observations discussed earlier from the in vivo experiments where the mean survival times of stored cells were significantly reduced and so this test could be of immense value as a means of identifying viable preparations.
9. CONCLUSION AND SUGGESTIONS FOR FURTHER WORK.

In conclusion the work presented has demonstrated the use of the encapsulation technique as a tool for examining membrane characteristics in relation to the treatment of sickle cell disease with Lys-Phe. The clinical observation that the dipeptide was relatively ineffective in vivo was undoubtably due to its inability to cross the membrane. By employing the encapsulation technique to introduce Lys-Phe into the cell, the predicted interaction between HbS and the dipeptide occurred.

Also demonstrated was the ability of erythrocytes to act as an efficient slow release system for the gastric acid secretion inhibitor, urogastrone. This illustrates a use of erythrocytes where although the rate of release is slow, the method is still applicable for compounds with high biological and pharmacological activity.

These observations have extended the successful use of erythrocytes as encapsulation vesicles. Although progress was achieved in storage, clearly further progress must be made before erythrocytes containing encapsulated materials can be commercially produced. An ideal system would be to freeze-dry preparations but obviously more research is needed.
Other areas for further work include a greater knowledge of the biochemistry as the cell ages. Also the pore or "tear" size of the expanded membrane should be determined more accurately. Sizing may be possible with the new range of nanoparticles recently available on the market. The problem of using electron microscopy to determine pore size in fixed preparations is in distinguishing artifacts from reality. The entrapment of nanoparticles containing entrapped drugs may also solve the problem of small molecules (e.g., drugs) "leaking" out of the erythrocytes too quickly for their use as a slow release system in vivo.

Other areas for research would be the separation of young cells from old cells on gradients, therefore increasing the survival times in vivo.

Finally, the successful use of the technique in treating animal models may help to promote more research and lead to the eventual use of the method in the clinical situation.
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