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ENCAPSULATION OF DRUGS IN INTACT ERYTHROCYTES: AN INTRAVENOUS DELIVERY SYSTEM

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Abstract—A number of drugs and the plasma antiprotease alpha$_1$-antitrypsin has been encapsulated in intact erythrocytes after hypotonic swelling, using a technique designed to preserve the viability of the cells. By labelling the cells with fluorescein isothiocyanate it has been shown that the cells survive exceptionally well when returned to the animal's circulation. Cell survival has been demonstrated in the rat, rabbit and guinea-pig. With encapsulation of cortisol-21-phosphate and methotrexate it was found that blood levels of the drug were maintained for a longer period than when the free drug was administered. Cortisol-21-phosphate was hydrolysed enzymatically by acid phosphatase located primarily in the erythrocyte membrane. An *in vitro* test involving the interaction or erythrocytes with phagocytes was developed to determine the viability or erythrocytes after being subjected to the encapsulation process. Preparations which did not interact with phagocytes survived when returned to the animal's circulation. The encapsulation procedure increased the fragility of the cell membrane compared to that of normal cells as measured by the leakage of haemoglobin after thermal treatment but it was found that encapsulated cortisol-21-phosphate in cells actually stabilized the membrane. The electrical charge on the membrane of encapsulating cells was the same as that of the normal cells. The charge on reformed ghosts was lower than that of normal cells. Reformed ghosts were rapidly removed when introduced into the circulation. The encapsulation procedure and its possible applications are discussed.

The systemic administration of free drugs to patients in certain circumstances suffers serious disadvantages. The drug may undergo premature degradation, inactivation or excretion with the consequent loss of pharmacological activity. Alternatively it may be necessary to protect the host from unwanted immunological or pharmacological effects.

Attempts have been made to overcome these problems by encapsulating drugs in microcapsules for use *in vivo*. Both synthetic and biosynthetic materials have been used in the preparation of microcapsules. Synthetic materials used include polyamide, polyurea, polyurethane, epoxy resin, ethyl cellulose, polystyrene, silicon rubber and gelatin [1, 2]. Problems encountered with the use of synthetic materials include the inability of the body to biodegrade many of the materials used and a failure to produce capsules small enough to clear the microcirculation. Biodegradable materials include the well-known 'liposomes' [3] which have a synthetic membrane modelled after natural membranes and polylactic acid which although it is a synthetic material, is biodegradable [2]. Attempts have been made to use the bodies own cells as drug carriers. Particular attention has been paid to erythrocytes in this capacity [4]. The major problem encountered in the use of biodegradable materials or natural cells as drug carriers is that they are removed *in vivo* by the recticulo–endothelial system. This seriously limits their useful life as drug carriers and in some cases may pose toxicological problems.

In this paper we have described a method whereby erythrocytes may be loaded with drugs or proteins without prior lysis of the membrane. The survival of the preparation *in vivo* in several animal species has been investigated together with biochemical properties of the encapsulating erythrocyte.

MATERIALS AND METHODS

In vivo *experiments involving animals*

Encapsulation of drugs in erythrocytes. The entire loading procedure was carried out under aseptic conditions at room temperature. Particular care was taken to avoid physical damage to the cells. All movements were gentle and transfers by pipette were done slowly. Blood was obtained from the animals under anaesthesia by cardiac puncture using a heparinized syringe. The blood was used as soon as possible after withdrawal. It was centrifuged at 600 *g* for 7 min and the plasma and white cells discarded. A portion (1 ml) of the packed cells was transferred to a test tube and 4 ml of a modified K^+-reversed Hank's solution was added. The modified Hank's solution consisted of KCl 10.18 g/l, KH_2PO_4 0.1 g/l, $NaHCO_3$ 1.273 g/l, NaCl 0.316 g/l, Na_2HPO_4 $2H_2O$

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0.1 g/l and glucose 2.0 g/l. This eutonic solution was diluted with distilled water so that its tonicity was 0.67 that of the cells. The cells and the solution were mixed by gently inverting the stoppered test tube several times. The suspension was centrifuged at 600 *g* for 5 min and the supernatant removed leaving a noticeably larger volume of cells. This volume increase was about 13% as measured by haematocrit. During the centrifugation a further portion of packed cells was lysed by the addition of an equal volume of water and 0.2 ml of this lysate was gently layered on top of the swollen cells. This layer acted as a barrier between the cells and the aqueous solution of the drug to be encapsulated, allowing transfers to occur with minimum chemical and osmotic shock to the cells. In addition the haemolysate provided an environment containing intracellular and cell surface materials which might otherwise be lost during the encapsulation procedure. An aqueous solution or aqueous hypotonic buffer solution of the drug to be encapsulated was prepared and a 0.2 ml 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 500 g for 3 min. The supernatant was retained as a barrier and the next 0.2 ml portion of the drug was layered on to its surface. The contents of the tube were mixed and centrifuged as before. Successive 0.2 ml portions of the drug were added until the erythrocytes reached the point of haemolysis. This point was apparent from a sudden increase in the transparency of the suspension, a darkening of the supernatant layer and a poorer separation on centrifuging with the appearance of a few white 'ghost' cells at the boundary between the cells and the supernatant. At the point of haemolysis the erythrocytes were resealed by the rapid addition and mixing with the calculated amount of modified K^+-reversed Hank's solution at 10 times eutonic strength. In practice this volume was approximately one-tenth of the total volume of the water added during the swelling and encapsulation procedure. The resealed cells were centrifuged at 500 *g* for 5 min and the supernatant discarded.

The cells were washed by suspending them in 10 ml of eutonic modified K^+-reversed Hank's solution followed by centrifuging at 500 *g* for 5 min. After discarding the supernatant the washing procedure was repeated three times to recover packed erythrocytes encapsulating the drug. For intravenous administration the packed cells were suspended in an equal volume of normal saline (B.P.) for injections. The process of encapsulation took about two hours and the preparation was used within six hours. In some experiments it was necessary to distinguish the cells used for encapsulation from normal cells *in vivo* (e.g. in tail vein smears). The packed cells prior to injection were suspended in 0.6 vol. of eutonic modified K^+-reversed Hank's solution saturated with fluorescein isothiocyanate (FITC) and allowed to stand for 1 hr at room temperature before washing and suspension in saline as described above.

Drugs encapsulated. The following drugs were encapsulated—insulin, alpha-1-antitrypsin, cortisol-21-phosphate, methotrexate and cyclophosphamide. All materials were purchased from Sigma (London, England). $Alpha_1$-antitrypsin was also prepared from human plasma in our laboratories.

All drugs were dissolved in water with the exception of methotrexate which was dissolved in 0.01 M $NaHCO_3$ solution.

Assay systems used for drug analysis. Cyclophosphamide was determined by gas-liquid chromatography [5] on a Pye series 104 instrument with a flame ionization detector. Methotrexate was determined by its inhibiting action on dihydrofolate reductase [6]. $Alpha_1$-antitrypsin was determined either by its trypsin inhibiting capacity [7] or by rocket electrophoresis [8]. Human $alpha_1$-antitrypsin was prepared from human plasma [9] using disulphide exchange chromatography on activated thiol Sepharose 4B (Pharmacia Fine Chemicals, Sweden). This $alpha_1$-antitrypsin proved to be more active than a sample purchased from a commercial source (Sigma Ltd.). Cortisol was estimated by a fluorimetric assay [10] or by radio-immunoassay using a commercial kit ('Cortipac S.C.6 kit, Radiochemical Centre, Amersham, England). Cell samples were lysed by freezing and thawing and solubilised by a solution consisting of 0.1 M Tris-HCl buffer (pH 7.6), containing 5 M urea, 3% w/v sodium dodecyl sulphonate, 0.1% v/v mercaptoethanol and 0.001 M EDTA. Usually cortisol was extracted from lysed and solubilized cells by chloroform. Cortisol-21-phosphate was hydrolysed prior to extraction with 1% w/v alkaline phosphatase at pH 7.4 for 1 hr at 37°. In one experiment an attempt was made to estimate cortisol-21-phosphate after enzymatic hydrolysis by estimating inorganic phosphate [11]. Steroids were also assayed, after extraction, by their ultra-violet absorbance in alcohol (19 : 1 v/v in water). ^{125}I-Insulin was added to soluble insulin (Sigma) as a tracer and the radioactivity determined in trichloracetic acid precipitated protein [12].

Osmotic pressure changes during encapsulation. The changes in osomotic pressure during the encapsulation procedure were measured by vapour pressure determinations on a osmometer (Knauer, Berlin). Samples of the supernatants obtained during the encapsulation procedure were removed by a capilliary tube and used directly for the determinations.

The survival of cells used for encapsulating drugs in vivo. Preliminary experiments established that pooled blood from our rat stock produced identical results to experiments where the rats own blood was used for the encapsulation procedure. For most rat experiments pooled blood from a number of animals was used as a matter of convenience. Our rats were male Wistar strain (Bantin and Kingman, Hull, England) of 200–300 g wt. Guinea-pigs were male Dunkin–Hartley strain of 300–350 g wt. (Bantin and Kingman) and rabbits were male Old English strain of 1.5–2.0 kg supplied by Fisons Pharmacological Laboratories (Loughborough, England). All animals were put under total anaesthesia by 3% halothane in oxygen and nitrous oxide for injections. They were allowed food and water *ad libitum* during all experiments. Rats and guinea-pigs were injected *via* the femoral vein and rabbits *via* the ear vein. Cells subjected to the encapsulation procedure but with drugs omitted were tested for survival *in vivo*

together with cells encapsulating cortisol-21-phosphate, cyclophosphamide and methotrexate. All cells used for the encapsulation procedure were labelled with FITC and injected slowly into the vein over 30 sec. Usually 0.5–1 ml of packed cells were injected into the animal's circulation. Immediately after the injection of the erythrocytes a tail vein blood sample was taken from the rats and ear vein samples from rabbits and guinea-pigs. The cells were counted using a standard haemocytometer grid but viewed under both ultra-violet and tungsten light (Zeiss Universal Microscope). By this method the percentage of FITC labelled cells in the circulation could be determined. Further blood samples were taken over the course of the experiment until FITC labelled cells could no longer be detected. For comparative purposes FITC was encapsulated in resealed erythrocyte ghosts using a modification of the technique employed by Tyrrell and Ryman [13]. Because of the low solubility of FITC in water the cells were treated with an equal volume of eutonic modified K^+ reversed Hanks solution saturated with FITC prior to complete lysis in water. After resealing the ghosts were washed in the same way as cells used in the encapsulation procedure and tested for survival *in vivo* in the rat.

Drug levels in vivo. Two experiments were performed to determine blood levels *in vivo*. In one experiment encapsulated cortisol-21-phosphate was administered by intravenous injection to rats and rats were bled by cardiac puncture in groups of four at intervals throughout the experiment. The cells were separated from the plasma by centrifugation and the amount of cortisol in both was determined by radioimmunoassay (Cortipac, Radiochemical Centre, Amersham, England) or by spectrofluorimetric assay [10]. In another experiment encapsulated methotrexate was administered to rats which were housed individually in metabolism cages which permitted the collection of urine free from faeces. The urine was assayed for methoxtrexate by its inhibitory action on dihydrofolate reductase [6].

In vitro *experiments*

The uptake of drugs by erythrocytes. In order to investigate whether the drugs were taken up by all cells during the encapsulation procedure an ultra-violet fluorescent derivative of methotrexate was synthesized [14]. This substance (F-MTX) was dissolved in 0.01 M $NaHCO_3$ (8 mg/ml) and encapsulated in rat erythrocytes by the standard procedure except that after mixing with each portion of the drug a small sample of the cells was removed with a capillary tube and a differential count made of fluorescent and non-fluorescent cells with the ultra-violet microscope. The cells were sealed at the point of lysis and after a final differential count the cells were separated on a bovine serum albumen gradient designed to separate cells according to their age [15]. Cell samples were removed from the various layers in the gradient and the percentage of ultra-violet fluorescent cells in each layer determined. In order to determine whether the drug had entered the cells normal erythrocytes were allowed to stand in contact with F-MTX in eutonic media. The cells after recovery were examined under the ultra-violet microscope as before.

Investigation of erythrocyte membrane charge by microelectrophoresis after various treatments. The electrophoretic mobility (which is a function of surface charge) was measured for rat and human erythrocytes which had received various treatments including encapsulation. The apparatus used was a Mark II microelectrophoresis apparatus (Rank Bros., Cambridge, England) with a cylindrical cell and platinum black electrodes [16]. The system was illuminated with a quartz iodine lamp and the scattered light observed by a camera fitted to a microscope fixed at the stationary level. Ten erythrocytes were timed electronically as they moved in the applied electric field over a fixed distance set by an eyepiece graticule and displayed on a TV monitor. Cells were timed with the field in both polarities to minimize errors due to drift. The cylindrical cell was filled with Sorenson's phosphate buffer (pH 7.4) and the erythrocyte suspension (1% v/v in saline BP) was gradually introduced to prevent bubble formation. The cell was flushed three times with buffer between different samples.

Electron microscopy of erythrocytes subjected to the encapsulation procedure. Rat cells were used in this experiment. The cells were swollen to the point of lysis by the usual procedure but the drug was omitted. The cells were not resealed at the point of lysis. Instead they were centrifuged down at 500 *g* for 3 min and the supernatant was removed. The cells were rapidly suspended in eutonic Hank's reversed K^+ solution (10% w/v) and dripped into a large excess of 2.5% w/v glutaraldehyde solution in Soreson's phosphate buffer (pH 7.4). The suspension was allowed to fix for 1 hr at room temperature and the cells were recovered by centrifugation. The cells were washed three times in Hank's solution and one drop applied to the aluminium carrier of the electron microscope. The procedure for fixing the red cells has been described [17]. The electron microscope used for these experiments was a Cambridge Stereoscope S150 instrument. Normal erythrocytes were also examined at the same time for comparative purposes.

Erythrocyte viability after encapsulation. Guinea-pigs and rabbits were used for these experiments. Polymorphonuclear leucocytes (PMN) were raised in the animals by the intraperitoneal injection of saline BP using 100 ml for guinea-pigs and 500 ml for rabbits. After six hours a further injection of saline was made and the exudate collected. Viability checks on PMN were made by trypan blue exclusion. To observe phagocytosis 30 drops of the exudate was mixed with 10 drops of homologous serum. The erythrocytes were diluted with three volumes of saline and one drop added to the PMN suspension. One drop of the final suspension was added to a cell made by supporting a coverslip on Sellotape on a microscope slide. The cell was examined by light microscopy using normal transmission and phase contrast.

The slow release of encapsulated drugs in dialysis sacs. Standard dialysis tubing-Visking (Medicell International Ltd., London, England) was used for these experiments. Small sacs 100 × 10 mm were

made and sealed at one end. A 0.5 ml portion of the material under test was added to the sacs. The sacs were suspended in 25 ml of medium at a depth of 50 mm using McCartney bottles as containers. Eutonic Hank's solution was used as the solvent or suspending medium throughout. The sacs were transferred to fresh medium every 30 min and the experiments were terminated after 3 hr. Each preparation was tested in five replicate sacs. A 1% w/v methylene blue solution was used as a standard and erythrocytes encapsulating alpha$_1$-antitrypsin and cortisol-21-phosphate together and cortisol-21-phosphate alone were tested against solutions of the free agents. In some experiments normal erythrocytes were added to the free drugs. Each bottle of medium was analysed for free cortisol and cortisol phosphate by its absorbance at 240 nm. The following sacs were set up: encapsulated cortisol-21-phosphate (1.34 mg), free cortisol-21-phosphate (3.55 mg), free cortisol-21-phosphate (3.55 mg) plus normal erythrocytes; encapsulated cortisol-21-phosphate (1.34 mg) with alpha$_1$-antitrypsin (4.2 mg); free cortisol-21-phosphate (3.55 mg) plus free alpha$_1$-antitrypsin (8 mg) plus normal erythrocytes and free cortisol-21-phosphate (3.55 mg) and alpha$_1$-antitrypsin (8 mg). The dialysis sacs prevented the diffusion of protein into the surrounding medium.

The interaction of cortisol-21-phosphate with erythrocytes

Stabilization of erythrocyte membranes. It is well known that corticosteroids stabilize biological membranes and the purpose of this experiment was to determine the possible effect of this action on the encapsulation of steroids in erythrocytes. Solutions of cortisol-21-phosphate were prepared in normal saline and 1 ml was mixed with 4 ml of a 1% v/v suspension of rabbit erythrocytes in saline to give a final steroid concentration in the range 10^{-7}–10^{-4} M. Duplicate samples were prepared and drugs were omitted from the controls. The tubes were incubated at 37° in a shaking water bath for 30 min. They were then centrifuged at 600 *g* for 10 min. The amount of haemoglobin in the supernatant was determined by its absorbance at 543 nm on a Pye Unicam spectrophotometer (SP500). Samples of encapsulated cortisol-21-phosphate and encapsulating media were set up in the same way. The original solution used for encapsulation contained 16 mg/ml of which 0.2 ml was used during the encapsulation procedure. Total haemoglobin was released by lysing the cells with 5 μl of Triton X100.

The hydrolysis of cortisol-21-phosphate by erythrocytes. Cortisol-21-phosphate is stable at room temperature in aqueous medium but the possibility that enzymatic hydrolysis may occur within the erythrocyte was investigated. Initially erythrocytes with encapsulated cortisol-21-phosphate were lysed by freezing and thawing and after centrifuging at 10,000 *g* for 10 min the supernatants were examined by thin layer chromatography (tlc) for free cortisol. Silica gel plates were developed in aqueous alcohol (95% v/v) and the steroid and its phosphate detected by spraying the plate with methanol, conc. sulphuric acid (1:1 v/v) and heating at 100° until the spots appeared. In a further experiment the membrane fraction of erythrocytes was separated from the cytoplasm by freezing and thawing followed by centrifugation at 10,000 *g* for 10 min. The cell free supernatant was retained and the membrane pellet was washed four times with saline to remove traces of cytoplasmic contents. The membranes were suspended in a citrate buffer pH 5.2 and the pH of the cytoplasmic fraction was also adjusted to pH 5.2 with the same buffer. The two fractions were incubated with aqueous solutions of cortisol-21-phosphate in a shaking water bath for 1 hr at 37° and after centrifuging the supernatants were examined by tlc for steroids.

Table 1. Amounts of drugs encapsulated in erythrocytes under various conditions

Drug	Solvent	Amount encapsulated mg/ml packed cells	% Encapsulated	Assay
Methotrexate	0.005 M $NaHCO_3$	0.150	6.2	Dihydrofolic reductase
Methotrexate	0.01 M $NaHCO_3$	0.611	15.5	Dihydrofolic reductase
Methotrexate	0.02 M $NaHCO_3$	0.243	6.3	Dihydrofolic reductase
Cyclophosphamide	Water	0.642	8.8	g.l.c.
Insulin	Water	57.6 mU	4.8	Radioactivity
Cortisol-21-phosphate	Water	2.90	18.0	Phosphate and spectrofluorimetric
Cortisol-21-phosphate	Water	2.09	14.6	Spectrofluorimetric
Cortisol-21-phosphate	Water	1.064	17.0	Spectrofluorimetric
Cortisol-21-phosphate	Water	0.77	11.6	Spectrofluorimetric
Alpha$_1$-antitrypsin	Water	4.20	5.0	Rocket immuno-electrophoresis
Alpia$_1$-antitrypsin	Water	10.0	20.0	T.I.C.

RESULTS

In vitro *experiments*

The encapsulation of drugs in erythrocytes. The results are given in Table 1 as the amount of drugs encapsulated per ml of packed cells and the percentage of the drug encapsulated of the amount added during encapsulation. Normal cells incubated with equivalent amount of drugs in eutonic media over the encapsulation procedure did not take up the drug. Therefore preswelling was necessary for the entry of drugs into cells. The omission of $CaCl_2$ and $MgSO_4$ in our modified K^+-reversed Hank's solution had no effect on the resealing and subsequent survival of the cells. It was omitted in the first instance due to problems with precipitation. The point of lysis was easily observed. Some variation between species was noted in reaching the point of lysis. Guinea-pig cells accepted about one third less water than rats before lysing. Rabbits cell accepted even less, about half, before lysing.

The amount of drug encapsulated varied with the concentration of the drug solution employed. Optimum conditions for the encapsulation of cortisol-21-phosphate were found to be when about 14–16 mg of cortisol (as the phosphate salt) was added to 1 ml of packed cells. When amounts in excess of this value were used the actual amount encapsulated fell (e.g. when 30 mg was added only 0.6 mg was encapsulated). Insulin posed a specific problem in that it was found that it was degraded by the cell. This was overcome by the addition of an inhibitor. The results concerning the encapsulation of insulin will be published separately.

The uptake of F-MTX by erythrocytes. The uptake of this methotrexate derivative is shown in Fig. 1. Clearly the cells only effectively take up the drug as the cells approach the point of lysis. Even at that point about 20% of the cells remain empty. When the cells were separated on a bovine serum albumin gradient [15] most of the FITC labelled cells were found in the upper less dense layer. The upper layer is normally associated with young less dense cells. Since our cells had been subjected to the encapsulation procedure it may be that the process itself modified the density of the cells. However gradient separation may offer a way of concentrating cells with encapsulated drugs. This would increase the drug loading in a unit volume of the cells.

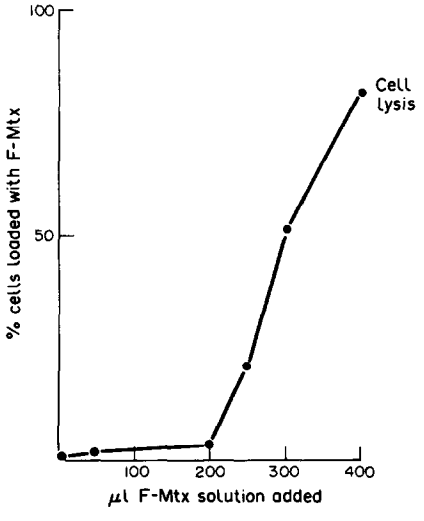

Fig. 1. The uptake of F-MTX during the encapsulation procedure. At the point of lysis 80% of the cells have taken up the compound.

Microelectrophoresis of erythrocytes. Fresh rat cells and cells aged for two hours, the normal length of the encapsulation procedure, did not show significantly lower mobilities U (μm/sec/V/cm) from cells subjected to the encapsulating procedure both in the presence and absence (sham encapsulation) of cortisol phosphate. The range of values was 1.815–1.769. Rat 'ghosts' gave a lower value of 1.757 ($P < 0.05$) compared to fresh cells. Human cells both fresh (1.508) and aged (1.581) gave lower values than rat cells which represents a species difference.

Electron microscopy. Plate 1 shows swollen cells fixed at the point of lysis. Numerous small protrusions were noted on the surfaces of the swollen cells. These were not produced when normal erythrocytes were subjected to the same fixing procedure. The presence of three echinocytes on the photomicrograph was probably due to contact with glass surfaces. Echinocytes were not found in preparations using normal erythrocytes.

Erythrocyte viability test by phagocytosis. This proved a very useful test. Within a few seconds of mixing the phagocytes with alien or damaged erythrocytes the phagocytes became very active in that the cells were very motile and pseudopodia appeared. Movement of the inner cell structure was very obvious under phase contrast. On contact with the erythrocyte the pseudopodia elongated until the red cell was completely engulfed. In the early stages of the engulfment the red cell was lysed. Individual phagocytes were found to engulf up to three red cells but the cells retained the ability to attach to and lyse further erythrocytes. Each engulfment took from three to five minutes and the reaction was complete within fifteen minutes. When the red cells were viable the phagocytes were not active. The combinations tested are listed in Table 2 and it was found that encapsulated cells with encapsulated cortisol-21-phosphate or alpha$_1$-antitrypsin were not attacked by phagocytes of their own species.

The slow release of encapsulated drugs from dialysis sacs. Non encapsulated cortisol-21-phosphate and methylene blue as expected diffused according to Fick's first law of diffusion. The addition of free alpha$_1$-antitrypsin or red blood cells did not affect the rate of diffusion. However when cortisol phosphate was encapsulated alone or with alpha$_1$-antitrypsin Fick's law was not obeyed and only about 3% of the total encapsulated drug had diffused out after 3 hr. The presence of encapsulated alpha$_1$-antitrypsin within the cell did not alter the diffusion rate.

Osmotic pressure and haematocrit determinations. These are summarised in Fig. 2. The osmotic pressure in the encapsulating environment was shown to fall to about half eutonic values at the point of lysis. The rise after the point of lysis was probably due to

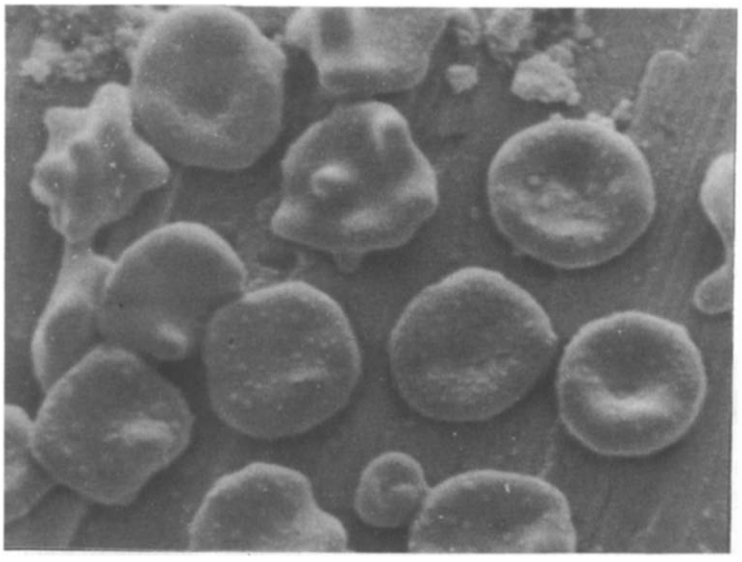

Plate 1. A photomicrograph of swollen cells fixed at the point of lysis. A feature of the preparation was the presence of numerous small protrusions on cells subjected to the encapsulation procedure. These protrusions were not found on normal erythrocytes subjected to the same fixing procedure.

the loss of intracellular components to the extracellular environment. The haematocrit value at the point of lysis was 123% of the normal value for packed cells. This is not necessarily a true indication of the volume increase of the cells since the packing of the cells would change as the cell shape changed from biconcave to spherical. The haematocrit value during encapsulation fell sharply when cell lysis occurred. Haematocrit or osmotic pressure determinations are therefore alternative methods for determining the point of lysis in the encapsulation procedure.

The stability of erythrocytes after the encapsulation of cortisol-21-phosphate. When normal cells were incubated at 37° the amount of haemoglobin released into the supernatant was adjusted to a 100% value. When cells subjected to the encapsulation process were tested in the same way the haemoglobin released gave a value of 217 indicating that some destabilization of the membrane had occurred. However when cells encapsulating cortisol-21-phosphate were tested the haemoglobin released was 41 ± 2% of the control, i.e. more stable than a normal cell. The effect of cortisol-21-phosphate at various concentrations on the rabbit erythrocyte membrane is shown in Fig. 3. At the lowest steroid level the red cells possessed about the same stability as normal cells but the stability increased up to an optimum concentration of steroid. Over this range the destabilization induced by the encapsulation process is countered by the stabilizing action by the drug itself. At the highest level the steroid was destabil-

Table 2. The interaction of PMN's with erythrocytes

Polymorphonuclear leucocytes	Erythrocytes	Reaction
Guinea-pig	Rabbit encapsulating alpha$_1$-antitrypsin	+
Guinea-pig	Rabbit encapsulating cortisol-21-phosphate	+
Rabbit	Guinea-pig	+
Rabbit	Rabbits own cells	−
Rabbit	Other rabbits cells	−
Rabbit	Rabbit encapsulating alpha$_1$-antitrypsin	−
Rabbit	Rabbit encapsulating cortisol-21-phosphate	−

A positive reaction was obtained when the PMN's lysed or engulfed the erythrocytes. In all cases the reaction proceeded with considerable vigour in contrast to negative reactions where the PMN's were totally inactive. Viability tests by trypan blue exclusion showed that 100% of the PMN's were viable in the peritoneal exudates.

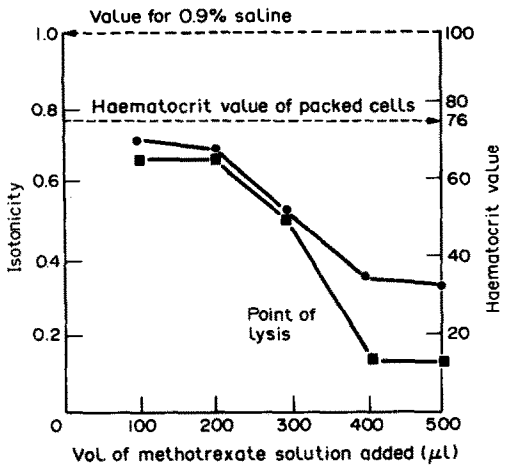

Fig. 2. The osmotic pressure and haematocrit values during encapsulation. Packed cells 0.5 ml were used in these experiments. Separate haematocrit values showed that the volume of normal cells increased by 23% at the point of lysis. The values on the graph were made during an encapsulation experiment starting from when the cells were preswollen with 0.67 × isotonic Hank's solution. They are not adjusted for volume changes. The point of lysis was judged by visual observation. Osmotic pressure, ●; haematocrit value, ■.

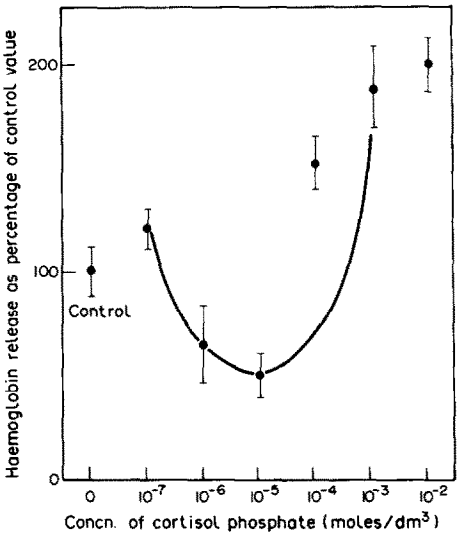

Fig. 3. The stabilization of erythrocytes *in vitro* by cortisol-21-phosphate. Stability was measured as a percentage of the rate of loss of haemoglobin where control values were adjusted to 100%.

izing. The biphasic stabilizing-labilising action of steroids on membranes has been established [19, 20].

The hydrolysis of cortisol-21-phosphate by erythrocytes. Lysed erythrocytes which had encapsulated cortisol-21-phosphate gave positive spots for cortisol after tlc using silica gel plates. Separate incubations with membrane preparations and cytoplasmic preparations also split cortisol-21-phosphate to free cortisol. However the cytoplasm produced less free cortisol than the membrane indicating that the bulk of the acid phosphatase activity was in the cell membrane.

In vivo *experiments*

The survival of cells used for encapsulating drugs

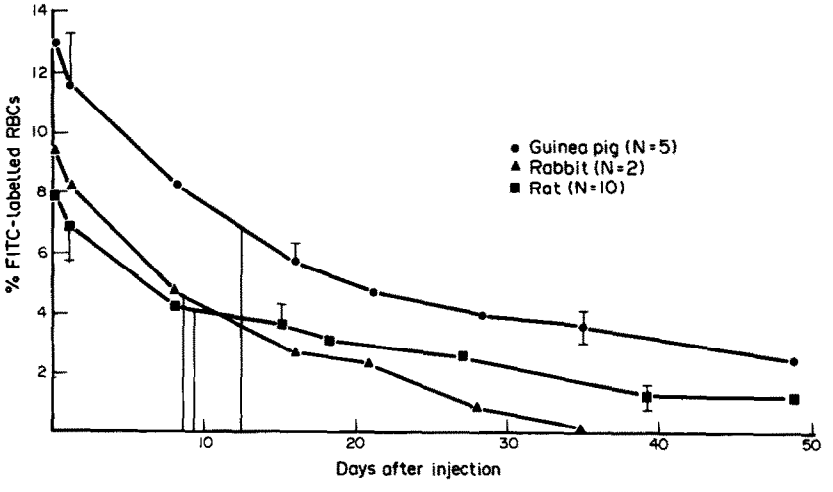

Fig. 4. The survival in the circulation of returned cells in the guinea-pig, rat and rabbit. All cells were subjected to the encapsulation procedure described in the text (sham encapsulation). The initial percentage on day 0 is the percentage of labelled cells *in vivo* immediately in the circulation after injection. All cells injected into the animals were labelled externally with FITC. Therefore the results represent the % of labelled cells in the total blood volume. The vertical lines represent $T_{\frac{1}{2}}$ values when half of the reinjected cells had disappeared. The values were 12 days (guinea-pig), 8 days (rat and rabbit).

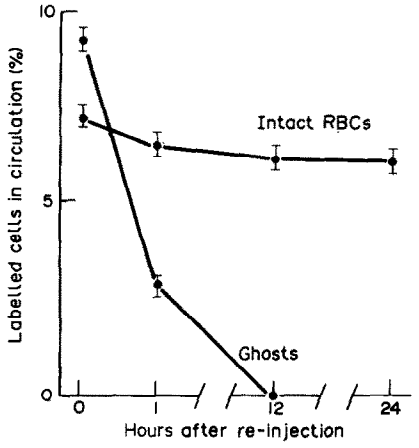

Fig. 5. The survival in the circulation of the rat of sham encapsulated erythrocytes compared with that of reformed ghosts. All cells were labelled externally with FITC. Details are given in the text.

in vivo. The survival of cells subjected to the encapsulation procedure for three species is shown in Fig. 4. The values on day 0 represent the percentage of labelled cells introduced into the animals circulation. Identical survival patterns were found in the rat when pooled cells from the same species were used instead of the rats own blood. Similarly the survival of the cells was not affected whether the cells were labelled externally with FITC or both internally and externally when FITC was incorporated into the encapsulating solutions. Our standard procedure used the external labelling technique.

In Fig. 5 the superiority of the technique compared to that of reformed erythrocyte ghosts is demonstrated since three quarters of the administered ghosts had disappeared within 2 hr and all had been removed by 12 hr. In contrast cells which had been subjected to the encapsulation procedure were still surviving after 24 hr when the experiment was terminated. It was noticed during the encapsulation programme that as workers became experienced with the technique the initial losses of cells in the circulation tended to diminish. This improvement was not found in experiments with ghosts. When drugs were encapsulated in rat cells (Fig. 6) the 50% survival time ($T_{\frac{1}{2}}$) of the preparations were similar to those obtained when the drugs were omitted (sham encapsulations). $T_{\frac{1}{2}}$ values were sham encapsulations (8–11 days) cortisol-21-phosphate (11 days) methotrexate (11 days) and cyclophosphamide (9 days). Labelled cells for all preparations were still observed in blood samples taken 50 days after the initial injections.

Drug levels in vivo. The urinary excretion patterns for methotrexate and the red cell and plasma levels for cortisol-21-phosphate are given in Tables 3 and 4. These show that the encapsulated preparations acted as a slow release system *in vivo*. With cortisol-21-phosphate a ten day period was chosen since this period was chosen as the therapeutic period for the treatment of adjuvant induced arthritis in the rat [18]. Survival curves for encapsulated cortisol-21-phosphate in the rat show that the half life for

Table 3. The amount of methotrexate in urine after rats were either treated with encapsulated methotrexate or free methotrexate i.v.

Urine (hr)	Total methotrexate μg/24-hr		
	Sham encapsulation	Encapsulation	Free
24	2	130	231
48	1	103	150
72	3	46	98
96	1	44	16
120	1	42	3

Urines were 24 hr urines collected at 10.00 hr each day.

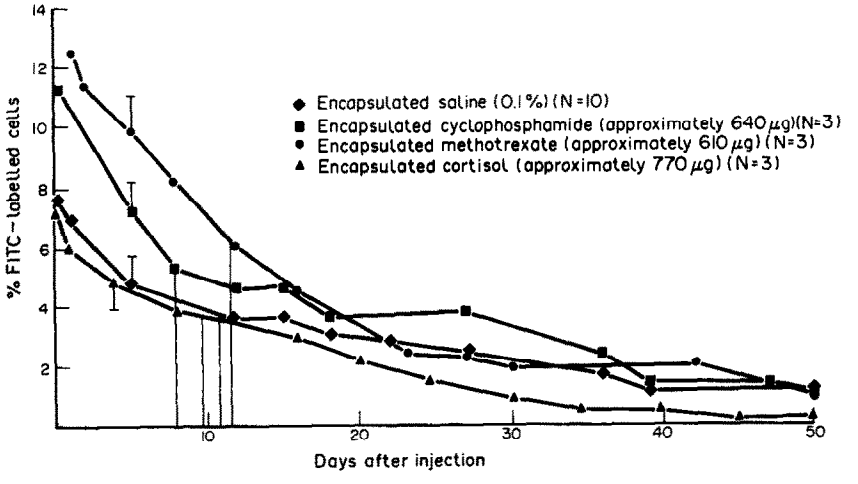

Fig. 6. The survival in the circulation of the rat of encapsulated cells containing various drugs. All cells were labelled externally with FITC. Details are given in the text. The values given represent the % of labelled (FITC) cells in the total blood volume. The vertical lines represent $T_{\frac{1}{2}}$ values. These were 9–11 days.

Table 4. The amount of cortisol in erythrocytes and plasma *in vivo* after the injection of packed cells encapsulating cortisol-21-phosphate

	Total cortisol in the blood of each rat (μg)					
	Assay (A)			Assay (B)		
Treatment	Cells	Plasma	Total	Cells	Plasma	Total
Normal rat cells	18	19.5	37.5	20.5	22	42.5
Expt. rat cells						
Day 0	546	16	562	—	—	—
Day 1	180	184	364	201	199	400
Day 3	164	163	326	171	166	327
Day 5	52	57	109	66	71	131
Day 10	45	27	72	61	52	113

Dose given was 1.064 mg per rat (Assay A) and 1.203 mg per rat (Assay B).

Rats were killed in groups of 4 after bleeding by cardiac puncture and the samples pooled for radioimmunoassay (A). For comparative purposes the experiment was repeated and single rats bled on days 1, 3, 5 and 10. In this experiment the samples were assayed by fluorimetric spectrophotometry (B). For calculation purposes the blood volume was assumed to be 15 ml.

the preparation is about ten days and the analysis confirmed that the blood levels of cortisol were still above normal ten days after the encapsulated preparation was administered. Both methods employed, radioimmunoassay and fluorimetric spectrophotometry, gave similar results for normal cells. The higher values obtained by the fluorimetric method in the experimental samples may include a contribution from metabolites since this method is less specific than radioimmunoassay.

DISCUSSION

The encapsulation procedure adopted in this study has permitted drugs to be encapsulated with minimum damage to the cells and showed that the cells escape removal by the recticuloendothelial system when returned to the circulation. This is in contrast to the majority of erythrocyte encapsulation systems, e.g. 'ghosts'. With corticosteroids it has been shown that effective anti-inflammatory blood levels of steroids are maintained for at least a fifth of the natural lifespan of the cell *in vivo* [18].

Preliminary work [21] on the encapsulation system was carried out at 0°. Although Passow [22] found that the resealing of erthrocytes was much slower at 0° than at 25° or 37°, we found difficulties when working at 0°. The most serious problems was that the cells would not survive when returned to the circulation. We also found that at 0° the viscosity of the cell suspension was so increased as to cause difficulties in mixing. Working at ambient temperatures not only enabled the cells to survive *in vivo* but preserved the membrane charge at normal value and the resistance of the cell to phagocytosis by polymorphonuclear leucocytes from its own species. The 'shelf life' of the preparation was not investigated but it is possible that storage at low temperature may prolong the viability of the preparation prior to use.

Larger volumes of blood may be used for encapsulation. We used 1 ml volumes for encapsulation as a matter of convenience for working with small laboratory animals. In some experiments we used 5 ml volumes without encountering difficulties. Very large volumes of blood may give problems in mixing the various constituents. The simplicity of the technique allows for modifications. For example a standard plastic blood transfusion bag might be used as a working vessel to minimize handling and possible contamination. Valve systems are available commercially to make the various additions and removals and the bag itself can be centrifuged at the relatively low speeds required for the technique.

The use of visual observation to judge the point of haemolysis was used for convenience. It is possible to predict quite accurately the amount of water that must be added in order for the cells to reach the point of lysis. This can be done experimentally by carrying out an osmotic fragility test or the haematocrit value or osmotic pressure can be monitored. Alternatively since there is a distinct visible change in the appearance of the cell suspension at the point of lysis other parameters such as refractive index changes or a simple colour chart might be used.

The method of entry of the drugs into the cells is unknown. Evidence presented by Seeman [23] showed that the permeable state of the cells existed only between 15 to 25 sec after the onset of haemolysis. Large particles such as ferritin or colloidal gold entered the cells which suggests that pores opened in the membrane. Much of the evidence for the existence of pores in the erythrocyte membrane is based on work carried out on erythrocyte ghosts [23, 24]. The experiments with F-MTX in our present work show that the changes in the permeability of the membrane to drugs is most marked at the point of lysis when the cell is at its maximum volume and it may be that pores or tears occur when the membrane is fully stretched. Electron micrographs of transient holes fixed in the open position by glutaraldehyde in human erythrocytes have been published

[25]. Many substances have been entrapped directly into reformed erythrocyte ghosts [4] but other methods have been reported for including materials to enter cells. These include the use of dialysis against hypotonic media [26] dielectric breakdown [27] electrically induced permeability [28] and amphotericin-B treated cells [29]. The disadvantage of the majority of encapsulation methods is that the cells lose their viability when returned to the circulation.

Our experience was that from 5–20% of alpha$_1$-antitrypsin added could be encapsulated and from 10–20% of added cortisol-21-phosphate dependent upon the concentration of the drug solution added. These figures are 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. It has been reported that ghosts under optimum conditions can take up between 40–50% of added drug [25]. It is doubtful that we can reach this degree of loading in our system because the experiments with F-MTX showed that at least 20% of the cells remained empty at the point of lysis. Enrichment of loaded cells by separation on gradients is a possibility but this approach needs further investigation.

The stabilization of the erythrocyte membrane by corticosteroids was of interest and it may be worth while to examine these cells for a prolonged survival time over that of normal cells. The degradation of the phosphate esters within the cell suggests the possibility of the use of such compounds as depot substances. The hydrolysis of these polar compounds could be used to release non-polar lipophilic substances which can diffuse through the cell membrane to the extracellular environment.

The encapsulation of insulin in erythrocytes has recently been published [30].

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