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ENZYME LOADING OF ELECTRICALLY HOMOGENEOUS HUMAN RED BLOOD CELL GHOSTS PREPARED BY DIELECTRIC BREAKDOWN

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SUMMARY

Human red blood cell ghosts were prepared by electrical haemolysis at 0 °C in isotonic solutions using a discharge chamber which was part of a high voltage circuit. The size distribution of the ghosts was normally distributed, the modal (=mean) volume was approx. 115 μm^3, performing the electrical haemolysis in the following solution: 105 mM KCl, 20 mM NaCl, 4 mM $MgCl_2$, 7.6 mM Na_2HPO_4, 2.4 mM NaH_2PO_4, 10 mM glucose, pH 7.2.

Resealing was carried out at 0 °C for 10 min (after the haemolytic step) and then for further 20 min at 37 °C.

The mean volume of the ghost preparation could be changed by variation of the phosphate concentration in the above solution replacing a part of NaCl by phosphate (5 mM phosphate: 94 μm^3, 15 mM phosphate: 134 μm^3).

The breakdown voltage of the ghost cell membranes measured with a hydrodynamic focusing Coulter Counter depends on the mean volume (94 μm^3 = 1.04 V, 134 μm^3 = 1.36 V). On the other hand, the breakdown voltage is constant throughout each size distribution pointing to an "electrically homogeneous" ghost preparation. The sensitivity of the Coulter Counter to detect electrical inhomogeneities in the membranes of a ghost population is demonstrated by dielectric breakdown measurements of an apparently normally distributed ghost preparation containing two different "electrically homogeneous" ghost populations i.e. with two different breakdown voltages. The ghost cells obtained by electrical haemolysis in the above solution containing 10 mM phosphate were fairly impermeable to sucrose and behave like an ideal osmometer.

It is further demonstrated that ghost cells can be loaded with enzymes (e.g. urease) and drugs using this technique and that these loaded ghost cells can be used as bioactive capsules for clinical application.

INTRODUCTION

The study of red blood cell ghosts has greatly extended our knowledge of the structure and function of cell membranes. Recently, ghost cells have attracted further

interest in clinical research. As suggested by Zimmermann [1–3] and later independently by Ihler et al. [4] red cell ghosts loaded with enzymes, hormones and drugs can be used as bioactive capsules in blood circulation without pronounced immune response. Both membrane research and microencapsulation of drugs in red cell ghosts for treating diseases require a preparation method by which homogeneous ghost cells are obtained in high yields.

Furthermore, for a broad clinical application a method is desired which allows the formation of ghosts with appropriate membrane permeability properties needed for a given clinical therapy. For the preparation of ghosts the procedure employing haemolysis in hypotonic solutions for the removal of haemoglobin has been used [5–7]. The disadvantage and limitations of this method, particularly the clinical applications, are well known. Therefore, we have been forced to use an electrical haemolysis method [8–12]. It is based on the dielectric breakdown of the cell membrane. Dielectric breakdown takes place in a local area of the cell membrane [13–15] when a cell suspension is exposed to high electric field strengths of the order of 10^3 to 10^4 $V \cdot cm^{-1}$ for a period of 1–50 μs.

Experimentally these conditions can be realized in a hydrodynamic focusing Coulter Counter or in an electrolytical discharge chamber which is a part of a high-voltage circuit. Dielectric breakdown of the cell membrane leads to a dramatic but reversible permeability change of the membrane due to which haemoglobin is released from the red blood cells. As shown for bovine red blood cells [9] the lysed cells resealed and trapped radioactive labelled albumin in high concentrations. After complete resealing the ghosts showed again the dielectric breakdown phenomenon thus providing evidence that the electrical properties of the membranes of the resealed cells are restored to nearly the original values. The red cell ghosts so obtained were normally distributed. The advantage of this method is evident. The preparation can be performed in isotonic solution, the manipulations are very easy and can be carried out routinely. The method also offers the decisive advantage of preparing ghost cell populations of various membrane permeability properties. This is suggested by the investigations of the dielectric breakdown of membranes of various cells.

The present study focuses on the preparation of "electrically homogeneous" human red cell ghosts which have optimal properties for clinical application. It is shown that the human red cell ghosts obtained by this electrical method are fairly impermeable to sucrose and that these ghosts loaded with urease can be used for the continuous hydrolysis of urea to ammonia and carbon dioxide. Urease was studied because of the great effort made by several authors (see review by Zaborsky ref. 16) to use microencapsulated urease intra- and extracorporally.

Such urease-loaded ghost cells may have important applications in the treatment of kidney failure providing the problem of ammonium binding is solved; the latter seems possible in the near future [17].

METHOD AND THEORY

Human red cell ghosts were prepared from fresh human blood of apparently healthy donors. The washed red blood cells were suspended in solution I containing 105 mM KCl, 20 mM NaCl, 4 mM $MgCl_2$, 7.6 mM Na_2HPO_4, 2.4 mM NaH_2PO_4 and 10 mM glucose, pH 7.2. The suspension density was about 10^9 cells/ml. An aliquot

of this suspension was placed in an electrolytical discharge chamber. The experimental procedure for the dielectric breakdown of the cell membranes was described elsewhere [9, 10]. The temperature was usually adjusted to 0 °C. The peak electric field strength was 12 kV · cm^{-1} [18]; the pulse length (the time constant) was 40 μs. After dielectric breakdown the equilibration and resealing period was 10 min at 0 °C followed by an incubation period of 20 min at 37 °C. For some experiments this period was extended to 1 and 2 h, respectively (see Results). Then the cells were centrifuged at 6000 × g and suspended in the isotonic solution II containing 145 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 7.6 mM Na_2HPO_4, 2.4 mM NaH_2PO_4 and 10 mM glucose, pH 7.2. The temperature was 37 °C. The haemoglobin content of the resealed ghosts was about 5 % referred to the original value in the intact red blood cells measured with a Zeiss photometer at 415 nm. No attempt was made to remove the haemoglobin completely although this is possible.

For the investigation of the permeability properties of the resealed ghost membranes [^{14}C]sucrose (Amersham, approx. 500 Ci/mol) was added to solution I immediately after dielectric breakdown occurred. After the equilibrium and resealing period the cells were centrifuged, washed several times with solution II to which inactive sucrose was added to remove adsorbed [^{14}C]sucrose on the membrane surface. The complete removal of the adsorbed activity was controlled by measuring the radioactivity in the washing solutions. Then the cells were incubated in solution II at 37 °C and the retention of [^{14}C]sucrose in the ghosts was measured as a function of time.

The radioactivity was measured in a Tri-Carb liquid scintillation spectrometer (Packard Instrument) after dissolving the samples in Soluene 100 and Insta-Gel.

In a parallel run the cells were destroyed by high electric field strengths to prove if the [^{14}C]sucrose was really trapped in the ghosts. It could be shown that under these conditions the total amount of trapped radioactivity was released into the supernatant. The incorporation of urease (Boehringer, Mannheim, G.F.R.) in the ghost cells was performed in the same way as described for sucrose.

In a second set of experiments the degree of resealing was studied by incubating the ghosts in solution II and measuring the dielectric breakdown voltage of the ghost membrane in a hydrodynamic focusing Coulter Counter (AEG-Telefunken, Ulm, G.F.R.). This method is also described in detail in several publications [10, 18–20]. Therefore, only a brief outline is presented here in order to understand the test for the "electrical" homogeneity of the ghost cells. A Coulter Counter is an instrument for determining size distributions. Size is defined as the volume of the particle multiplied by the so-called shape factor, f_i, which is 1.5 for a spherical particle and 1.0 for an infinite long cylindrical particle. The volume distribution of a population of single cells can be calculated by calibration of the Coulter Counter channels with spherical Latex particles according to the following equation:

$$V_E = (f_L/f_E) \cdot V_L, \tag{1}$$

where $V_{E(L)}$ = volume, $f_{E(L)}$ = shape-factor. The subscript E refers to erythrocytes and ghosts and L to Latex particles.

It should be pointed out that for the calculation of the ghost volumes reported here the assumption was made that the ghost cells are deformed in the hydrodynamic focusing orifice of the Coulter Counter like the intact cells. Therefore, it seems

plausible to use the shape factor of 1.09 which was determined by Thom [21] for the intact cells.

When measuring the size distribution of the red blood cell or ghost suspensions dependent on increasing electric field strength in the orifice of the Coulter Counter (60 μm in diameter and length) a shift in size distribution is observed above a critical electric field strength. This shift is caused by dielectric breakdown of the membrane. Solving the Laplace equation for elliptical cells on the assumption of suitable boundary conditions the critical membrane voltage, V_c, can be calculated easily from the following equation:

$$V_c = f_i \cdot a_i \cdot E \tag{2}$$

where E is the maximum field strength in the orifice; a_i is the long semi-axis of an ellipsoid parallel to the electric field (or the radius of a sphere); and f_i is the corresponding shape factor.

The field strength was calculated from the current density in the orifice and the specific conductance of the medium used for Coulter Counter measurements. This is comparable to the field strength calculated from the potential difference across the orifice assuming that 60 ± 10 % of the applied potential appears across the orifice [18].

The critical membrane breakdown voltage, V_c, depends only on intrinsic elastic and dielectric properties of the membrane [9–11, 13–15] and therefore is independent of the volume of the particle in a given size distribution. Therefore, the critical external electric field strength is volume-dependent as indicated by Eqn. 2.

Even if the size distribution is normal the possibility exists that ghost cells have differing breakdown voltages due to differing degrees of membrane resealing, and, therefore, different membrane properties (see Fig. 4) (or due to different deformation, and, therefore, to different shape factors).

This can be easily checked by calculating the critical membrane breakdown voltage, V_c, for a certain volume, say, for the modal or mean volume, and using this value of V_c for the calculation of the critical external electric field strength for the remaining volumes of the distribution. If the ghost preparation is "electrically homogeneous" the theoretical curve of the dependence of the critical external electric field strength on the volume should coincide with the experimentally determined one [9, 10, 20].

RESULTS

Electrically homogeneous ghost cells

In Fig. 1 a typical size distribution of ghosts prepared by electrically induced haemolysis is presented together with the size distribution of the intact cells from which the ghosts were obtained. The red blood cells were haemolyzed at 0 °C in the discharge chamber containing solution I. After an equilibration period of 10 min at 0 °C the temperature of the solution was increased to 37 °C for 20 min, the size distribution was determined in solution II at room temperature dependent on the electrical field strength in the orifice of the hydrodynamic focusing Coulter Counter. Only the size distribution measured at an electric field strength of 0.95 kV $\cdot$ cm^{-1} (which is well below the breakdown field strength) is given in Fig. 1 for reasons of clarity. Note, that the ghost size distribution is normally distributed like the size

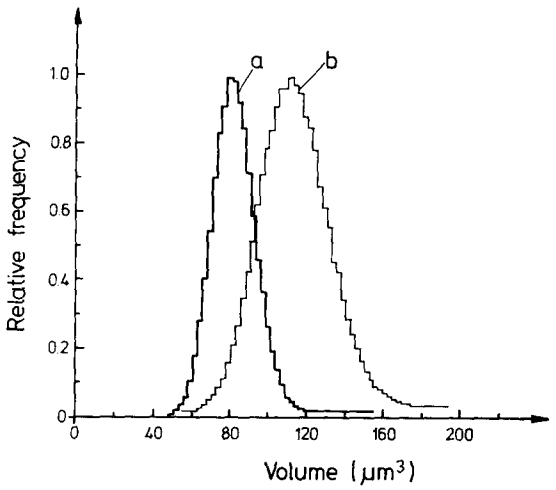

Fig. 1. Size distributions of human erythrocytes (a) and ghost cells (b) measured in a hydrodynamic focusing Coulter Counter. (Orifice: 60 μm in diameter and length; electrical field strength 0.95 kV · cm^{-1}.) The ghosts were prepared from the erythrocytes shown in this figure by dielectric breakdown in an isotonic solution which was placed in an electrolytical discharge chamber. The volume axis was calibrated by measuring Latex particles (d = 1.857 μm). The different shape factors of erythrocytes and ghosts (1.09) and of Latex particles (1.5) were taken into consideration.

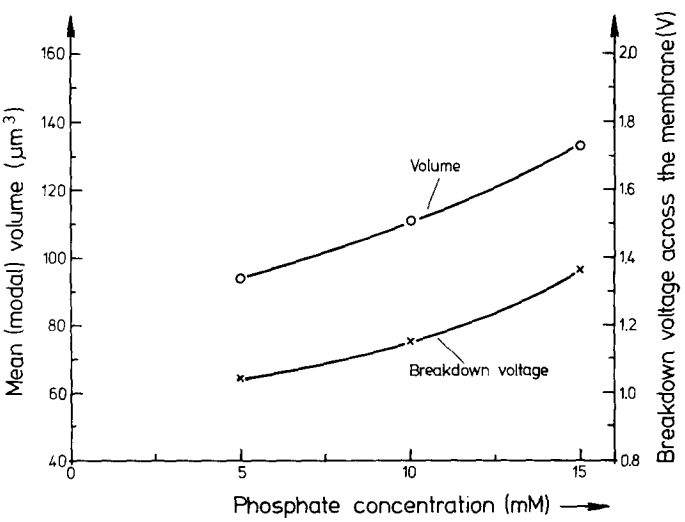

Fig. 2. The mean (modal) volume of normally distributed ghost populations (circles) prepared by electrical haemolysis is plotted against increasing phosphate concentrations in solution I containing 105 mM KCl, 4 mM $MgCl_2$, and variable NaCl concentration (depending on the phosphate concentration). Resealing was carried out for 10 min at 0 °C and for a further 20 min at 37 °C. As indicated in the figure the breakdown voltage of the cell membranes of the ghost cells (crosses) increases with increasing phosphate concentration.

distribution of the intact red blood cells. This is true also for the size distributions measured at various electric field strengths in the orifice. Cell fragments as described for the preparation of ghosts from bovine red blood cells [9] using this technique for the first time could not be detected although the resolution of the hydrodynamic focusing Coulter Counter is very high. The modal (= mean) volume of the ghost population is about 115 μm^3. However, the mean ghost volume depends on the phosphate concentration of the external medium during electrical haemolysis and resealing. Different phosphate concentrations in the range between 5 and 15 mM were achieved by replacing part of the sodium chloride in solution I by sodium phosphate. The tonicity was kept constant. Phosphate concentrations above 15 mM could not be investigated since higher phosphate concentrations suppressed the haemoglobin release (unpublished data). On the other hand, lowering the phosphate concentration below 5 mM results in skewed distribution of ghost populations. As indicated in Fig. 2 increasing the phosphate concentration results in increasing mean volumes of the normally distributed ghost populations. The dielectric breakdown voltage in each distribution also increases with increasing mean volume of the distribution (Fig. 2). As can be seen from Fig. 2 the breakdown voltage varies between 1.04 V (94 μm^3) and 1.36 V (134 μm^3). For comparison, the breakdown voltage of the intact red blood cells (mean volume = 83 μm^3) is 1 V.*

Prolongation of the equilibration (and resealing) periods in solutions I and II have no further pronounced effect on the breakdown voltage of the ghost membranes pointing to a complete and electrical reconstitution of the ghost membrane.

Although the breakdown voltages increase with increasing mean volume of the distributions, the breakdown for volumes of a given distribution is constant as predicted by the Laplace equation. This was tested as described above by using the breakdown voltage of the modal volume and calculating the critical electric field strength dependent on the volumes of a given distribution. In Fig. 3 the results are given for the size distribution with a mean volume of 115 μm^3, i.e. the size distribution which is obtained in solution I buffered with 10 mM sodium phosphate. The triangles present the theoretical curve using the value of 1.11 V calculated for the breakdown voltage of the membrane of the modal volume, whereas the squares present the experimentally-determined ones. The two curves coincide indicating that the dielectric breakdown voltage of the ghost membranes of volumes belonging to the same distribution is volume-independent. The same is true for the size distributions showing mean volumes between 94 μm^3 and 134 μm^3 obtained by the incorporation of different amounts of phosphate during the resealing process.

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* The breakdown voltage is a little smaller than that reported for human red blood cells previously [10]. It may be that the specifications of the manufacturer (AEG-Telefunken, Ulm, G.F.R) for the dimensions of the orifice vary within a limited range. This would falsify to some degree the calculations of the external electric field strengths in the orifice. However, the calculation of the critical external electric field strength and the assumptions for boundary conditions of the solution of the Laplace equation are so approximate due to the various time effects involved in the breakdown process [18] that in any case the absolute values calculated from Coulter Counter experiments must be considered critically (for a critical reappraisal of the exact connection between the breakdown and the membrane potential, see ref. 18). Fortunately, in most experiments only the changes, or the relative values, of the critical membrane potential are of interest.

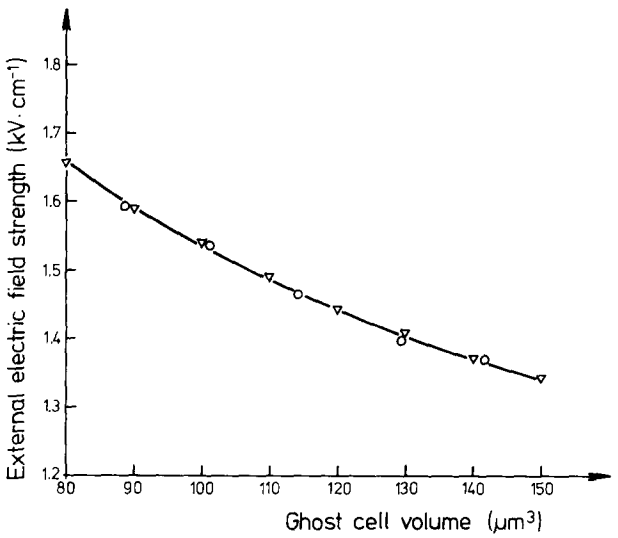

Fig. 3. The external electric field strength producing dielectric breakdown of the cell membrane of human ghost cells as a function of the volume. The triangles indicate theoretically calculated values of the external electric field strength, using Eqn. 2 and a critical membrane breakdown voltage V_c = 1.11 V; the squares indicate the external electric field strength experimentally determined from Coulter Counter measurements as shown in Fig. 1. Both curves coincide.

According to the considerations outlined above this finding points to a completely "electrically homogeneous" ghost preparation and, therefore, probably also to a ghost population with uniform membrane properties.

However, the question arises as to how sensitive "electrically inhomogeneous" ghost populations, i.e. ghost populations in which the membranes have different breakdown voltages (due to different membrane properties or different deformation), can be detected in a ghost preparation.

The sensitivity of Coulter Counter measurements in detecting electrically inhomogeneous populations in a ghost preparation is demonstrated in Fig. 4. Ghosts were prepared in solution I containing 10 and 15 mM phosphate, respectively. The mean volumes of the resulting ghost cell distributions were 120 μm^3 and 136 μm^3, respectively, and the corresponding breakdown voltages 1.17 and 1.32 V, respectively.

The two homogeneous ghost populations were mixed in a ratio 1 : 1 and the size distribution was measured dependent on increasing external electric field strengths in the orifice (0.5, 1.9 and 2.4 kV · cm^{-1}). The measurements were performed with compensated gain [16], i.e. the increase in pulse height with increasing field strength was compensated by an equivalent decrease in the electronic amplification. Therefore, in the absence of dielectric breakdown it would be expected that the size distribution curves measured at various electric field strengths in the orifice are identical if in each measurement the same number of cells are counted (100 000). This compensated gain procedure was chosen for clearness and for demonstration of the high resolution of the set-up. As indicated in Fig. 4, the size distribution appears normal in the subcritical range of the electric field strength in the orifice.

However, due to the difference in the breakdown voltage the size distribution

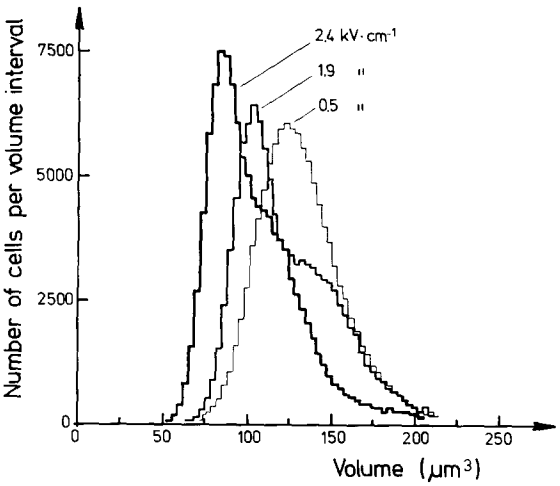

Fig. 4. The size distribution of an "electrically heterogeneous" ghost preparation was measured at three different electric field strengths (0.5, 1.9 and 2.4 kV · cm^{-1}) in the orifice. The measurements were performed with compensated gain. This means that the increase in pulse height dependent on increasing electric field strength was compensated by a corresponding decrease in the electronic amplification. The "electrically heterogeneous" ghost preparation was obtained by mixing two normally distributed and "electrically homogeneous" ghost populations in the ratio 1 : 1 (120 μm^3, 1.17 V and 136 μm^3, 1.32 V). As indicated by the figure, the size distribution which appears normal at low field strength shifts at higher field strengths towards smaller volumes (due to the dielectric breakdown) and simultaneously becomes very skewed. The reason for this skewness is that at 1.9 kV · cm^{-1} only the critical breakdown voltage of one of the size distributions being present in the heterogeneous preparation is reached.

shifts towards smaller volumes and becomes very skewed in the range of an external electric field strength (1.9 kV · cm^{-1}) at which one of the size distributions has reached the critical breakdown voltage. Thus we can conclude that the ghost preparations obtained by the electrical haemolysis are indeed electrically homogeneous in the range of accuracy of ± 0.05 V in V_c.

Impermeability of ghost cells to sucrose

The degree of restauration of the membrane permeability was also tested by measurements of the loss of [^{14}C]sucrose from loaded ghost cells.

In the experiments indicated by triangles and squares in Fig. 5 [^{14}C]sucrose was added immediately after dielectric breakdown in the discharge chamber containing solution I at 0 °C. In the experiments, denoted by squares in Fig. 5, the cells were kept in this solution for 10 min at 0 °C and 20 min at 37 °C. Following this the cells were centrifuged and washed as described above, and incubated in an inactive solution II in which the loss of [^{14}C]sucrose from the ghost cells was followed over several hours. In the experiments, denoted by triangles in Fig. 5, the equilibration and resealing periods in solution I at 0 °C and 37 °C were extended to 1 h and 2 h, respectively. Under both experimental conditions equilibration between the internal and external solutions was reached, i.e. 100 % of the external [^{14}C]sucrose trapped in the ghost cells. As shown in Fig. 5 the [^{14}C]sucrose loss from the resealed ghosts is very

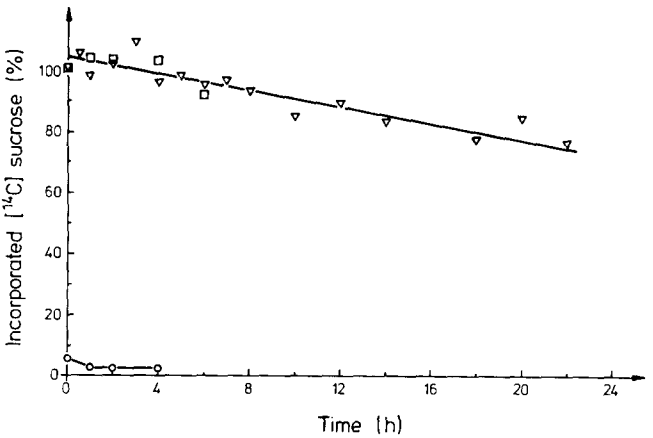

Fig. 5. Retention of incorporated [^{14}C]sucrose by human ghost cells as a function of time. The haemolysis was induced by dielectric breakdown of intact red blood cells in isotonic solution I in an electrolytical discharge chamber. The [^{14}C]sucrose was added immediately after dielectric breakdown. After different equilibration and resealing times (see below) the ghost cells were transferred to inactive solution II and the time course of the incorporated ^{14}C[]sucrose was measured. The equilibration and resealing varied as follows: ▽–▽, 1 h at 0 °C, 2 h at 37 °C; □–□, 10 min at 0 °C, 20 min at 37 °C. In a second experiment (○–○) ghost cells were incubated for 30 min in solution II containing [^{14}C]sucrose after an equilibration and resealing period of 10 min at 0 °C and 20 min at 37 °C in solution I and the time course of the incorporated radioactivity was measured. The incorporated [^{14}C]sucrose referred to the activity of the suspensions after addition of [^{14}C]sucrose. The data points are average values from 5 different sets of measurements.

slow and is independent of the equilibration and resealing periods indicating that the resealing process is very rapid. This is also supported by experiments (circles in Fig. 5) in which the [^{14}C]sucrose was added to ghost cells in solution II into which the cells were transferred after a period of 10 min at 0 °C and 20 min at 37 °C in inactive solution I. Under these conditions only 5 % of the external activity can be trapped in the ghosts. It should be noted that the [^{14}C]sucrose loss from the cells occurs steadily at a rate of about 1 % per h referred to the incorporated [^{14}C]sucrose. This value is also observed for ghosts prepared by osmotic haemolysis (Deuticke, B. and Haest, J. W. M., personal communication).

In a run of parallel experiments, in which the effect of temperature during dielectric breakdown and removal of haemoglobin from the red blood cells on the resealing properties was studied, the temperature of solution I in the discharge chamber was adjusted to 22 °C.

The increase in temperature reduced the incorporated amount of [^{14}C]sucrose only from 100 to 80 %; the loss from the loaded ghost cells is unaltered and is about 1 % per h.

The impermeability of the resealed ghost membrane against sucrose suggested by these experiments can also be demonstrated by osmotic experiments using inactive sucrose as an osmotic agent. Changing the osmolarity of solution II by adding sucrose and plotting the mean volume of each size distribution versus the corresponding reciprocal osmotic pressure in the external medium yields a straight line as predicted by van 't Hoff's law (Fig. 6). Therefore, we can conclude that the ghost cells behave like an ideal osmometer.

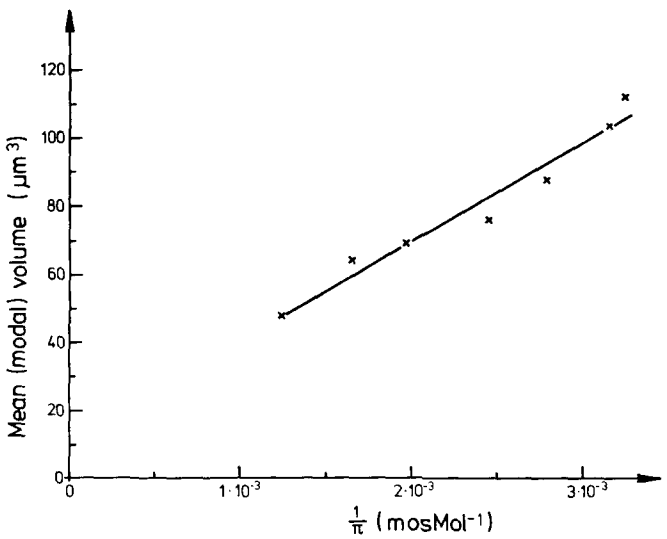

Fig. 6. The mean volume of normally distributed ghost cells is plotted against the reciprocal value of the osmolarity of the external solution II. Different osmolarities were achieved by addition of sucrose. The ghost cells were prepared as described in Methods and Theory.

Urease-loading of ghost cells

To get an initial insight into the behaviour of enzyme-loaded ghost cells the degradation of urea to ammonium and CO_2 was studied with ghost cells loaded with urease. The amount of ammonia set free by the urease trapped in the ghost cells was determined by the Berthelot reaction. The resulting blue colour of the Berthelot reaction was measured with great accuracy in a photometer at 550 nm. To detect a permeability change of the ghost cell membrane caused by the trapped urease over the time of the experiment [^{14}C]sucrose was simultaneously incorporated into the ghost cells. Electrical haemolysis was performed at 0 °C by applying an electric field of 12 kV · cm^{-1} for 40 μs. [^{14}C]sucrose and urease were added 5–10 s after application of the electrical field. After 10 min at 0 °C the temperature of the suspension was raised to 37 °C and kept for 20 min at this temperature as described above. The ghost cells were then centrifuged and washed 5 times with solution I to which 0.1 % inactive sucrose and 0.1 % albumin were added to remove adsorbed molecules on the external membrane surface. Control experiments have shown that under these conditions complete removal occurs. For these control experiments resealed ghosts containing no urease were suspended at 37 °C in solution II containing additional 0.1 mg urease/ml for 30 min. After centrifuging (4000 × g, 5 min) the ghost cells were washed 5 times as described above with solution I to which 0.1 % albumin was added. Investigations of the last wash water and ghost sediment showed that no urease activity could be detected.

The loaded cells were then divided into two aliquots. One aliquot was suspended in an isotonic solution containing no urea and the leakage rates of urease and [^{14}C]sucrose out of the cells were determined. The other aliquot was incubated in an isotonic solution containing 50 mM urea to determine the rate of ammonia produc-

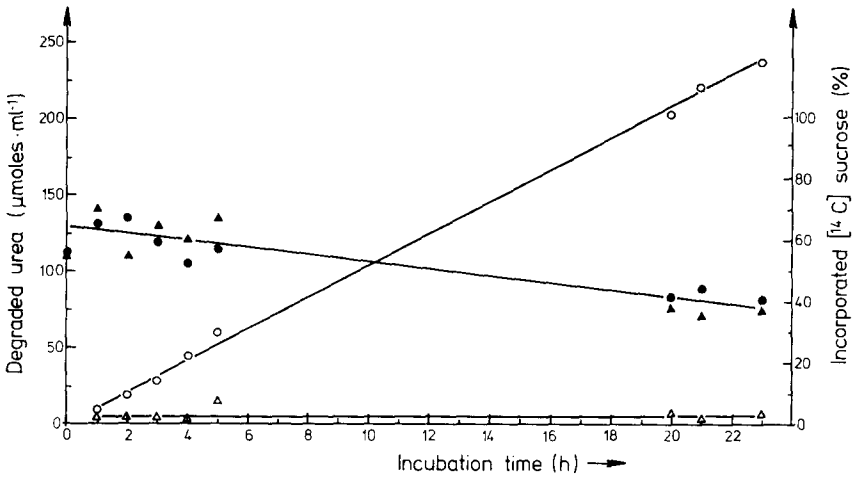

Fig. 7. Catalyzed hydrolysis of urea to ammonium and carbon dioxide by urease and [^{14}C]sucrose loaded ghost cells. In one set of experiments (○) ghost cells were suspended in solution II to which 50 mM urea was added. Since the reflection coefficient of the membrane to urea is very low, the mean volume of the ghost population changed only slightly. The analysis of the ammonium produced was based on the so-called Berthelot-reaction. The blue colour developed was measured photometrically at 550 nm. ●, denote sucrose loss observed during the experiment (measured in the sediment). In another set of experiments urease and [^{14}C]sucrose loaded cells were incubated in solution II containing no urea. ▲, denote the sucrose loss as a function of incubation time measured in the supernatant; △, denote the results of the urease analysis in the supernatant. It is obvious that urease has not passed the membrane and it is also evident from the figure that sucrose loss is not effected by the urea in the solution since ▲ and ● are on the same line.

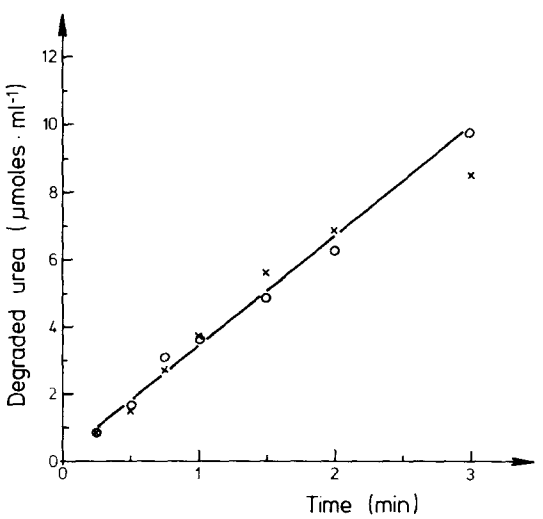

Fig. 8. The hydrolysis of urea catalysed by urease-loaded cells (crosses) and by urease loaded cells osmotically destroyed (circles). As indicated in the figure the hydrolysis of urea per time of the lysed cells is identical with that of the intact loaded cells suggesting that no restriction by the membrane to the movement of urea and no change in enzyme activity due to the microencapsulation has occurred.

tion during 24 h. The results are shown in Fig. 7. Zero time on the abscissa corresponds to the beginning of incubation of the ghosts in this isotonic solution. As indicated in Fig. 7 (filled triangles and circles) the sucrose loss is slightly higher over 24 h than in the absence of urease (Fig. 5), indicating that the membrane becomes a little more permeable to sucrose. Loss of urease is not observed (open triangles). The open circles show the catalytic production of ammonia by the urease loaded cells. The hydrolysis rate of urea is constant over 24 h. In some preliminary experiments constant rates could be observed over a period of 48 h and more. Therefore, the use of loaded cells for longer continuous operations seems possible.

The activity of the urease trapped inside the ghost cells was about 20 % of the activity of the urease present in the external solution during electrical haemolysis and resealing. This was shown by comparing the activity of the original suspension to that of the loaded ghost sediment. It may be argued that this result was falsified by the lowering of the rate of the enzymatic hydrolysis of urea by restriction by the membrane of the movement of urea or by a change in the enzymatic activity due to the encapsulation. This objection was tested by measuring in a parallel set of experiments the urease activity of loaded ghost cells to which distilled water had been added so that the cell membrane ruptured. The activity of the lysed cells was compared with that of the intact ghost cells. As indicated in Fig. 8 the amount of ammonia produced per min is the same in both cases. Therefore we can conclude that there is no restriction by the membrane to the movement of urea or change in the enzyme activity.

DISCUSSION

From the results reported here we can conclude that haemolysis induced by a transient change in the membrane permeability in response to high electric field strengths (dielectric breakdown) is a rapid and elegant method for preparation of ghost cells from human red blood cells in large quantities with high efficiency. If the phosphate concentration of the solution in which the electrical haemolysis was performed (solution I) is kept above 5 mM the ghost cells are normally distributed and "electrically homogeneous". The term "electrical homogeneity" was introduced to describe the volume-independence of the dielectric breakdown voltage for a given cell population as it is predicted by the Laplace equation for cells exhibiting homogeneous membrane properties. This means in terms of the electro-mechanical model (provided that the shape factor is identical for different cell sizes of the same distribution) [9, 10] that the transverse elastic or compressive modulus, the dielectric constant and the thickness of the membranes are identical for smaller and larger cell sizes.

The experiment described in Figs. 3 and 4 has presented convincing evidence that the dielectric breakdown is a sensitive tool for testing the homogeneity of a ghost preparation provided that the dielectric breakdown voltage of the membranes of different cell populations existing in the preparation are different. Since dielectric breakdown is also observed using bacteria [18] and algal cells [20] this technique may have a broader application in solving similar problems.

In this paper no attempt was made to reveal the functional behaviour of the electrically prepared ghost cells. From the purpose outlined above it seemed sufficient to demonstrate the restoration of the membrane permeability with respect to sucrose. Investigations of the function of the ghost cells provided at first a detailed study of the

influence of ions, particularly calcium and bivalent anions, on the electrical haemolysis and the resealing process. This view is suggested by the skewness of the size distribution of ghost cells observed in solutions containing low phosphate levels. This skewness, which may result from the existence of at least two ghost populations of different mean volumes and breakdown voltages, points to a specific effect of phosphate since a change in pH of the low buffered solutions during haemolysis and resealing could be definitely excluded.

In any case, although the question concerning the function of electrically prepared ghost cells is still open, there may be yet a considerable interest in performing biochemical, biophysical and electron microscopical studies with ghosts like those described here which were not subjected to a transient hypotonic stress. Since it is well known that solutions of low ionic strength solubilize membrane proteins [22, 23] there may exist differences in membrane structure and composition between osmotic and electrically prepared ghost cells.

Another important application of electrically prepared ghost cells was also demonstrated in this paper. The electrical method offers the opportunity to use loaded red blood cells for correcting metabolic disorders or for controlled drug release. We feel that the results obtained here are quite encouraging since the difficulties usually arising in immobilization of enzymes (or drugs) by microencapsulation can be overcome by the use of such loaded cells (see review, ref. 16).

Major contributions for the use of immobilized urease (but also of asparaginase and catalase) for intra- and extracorporal application were made by Chang et al. [24–28], Gardner et al. [29, 30], Sparks et al. [17] and several other authors [31–35]. Despite great difficulties involved in the use of microencapsulated enzymes and drugs the impressive results obtained by these authors justify future attempts in developing new techniques for microencapsulation. The method described here is exceedingly simple. The enzyme is trapped inside the membrane envelope. A substrate permeable to the membrane can transfer across the membrane and be transformed into the product. The product, in turn, can permeate across the membrane into the external phase. It is obvious, that with this method of immobilization, no changes in the intrinsic properties of the encapsulated enzyme are anticipated. Furthermore, it is certainly true to assume that an immune response does not appear or is greatly delayed, i.e. after recovery of the organism. Therefore, the use of loaded cells offers the possibility to hydrolyse or to degrade substrates intracorporally which may be of great interest in the treatment of diseases like kidney failure, acatalasaemia and some asparagine-dependent tumors or in controlled drug release in general.

The disadvantage of this method is the limitation to only enzymes, drugs and substrates which will not alter the membrane permeability drastically. Furthermore, the key problem for a long-term clinical application is also the stability of the loaded cells. At present, data concerning the stability of loaded cells in vivo are still lacking and the information which is obtained from experiments in vitro may be very limited. However, it can be expected that ghost cells exhibiting similar size and surface charge like intact red blood cells will be stable over a long period in the blood circulation.

On the other hand, ghost cells varying in size and surface charge can be used for short-term, organ-specific applications in controlled drug release, as suggested by Schmidt, F. W. and Schmidt, E. (personal communication) since ghost cells with a

mean volume and surface charge different to that of intact cells will be degraded specifically in the organism.

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