ize. The presence of NaCl during the fractionation does not interfere, since it is diluted by the addition of the saturated ammonium sulphate solution. The possibility of altering the fractionation pattern by the addition of a ligand specific for an enzyme or group of enzymes, as exemplified here with NADH for GDH and MDH, may be used to improve the purification attainable with ammonium sulphate alone.

We believe that the simple procedure outlined here can be useful as a general first step in the difficult task of the purification of halophilic enzymes[10].

Resúmen. Seis enzimas de la bacteria halófila extrema *Halobacterium cutirubrum* han sido parcialmente purificadas por fraccionamiento de los extractos crudos en ClNa 5 *M* con solución saturada de sulfato de amonio. Se discuten las ventajas del procedimiento, como un método general para enzimas halofílicas, comparado con otros métodos descriptos en la literatura.

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Determination of the Trapped Volume in a Pellet of Red Blood Cells

Separation of cells from a suspension by repeated centrifugation and resuspension, known as 'washing', is often inadequate. The volume trapped between sedimented cells after centrifugation is a measure of the degree of separation (of the cells) from the suspension medium. Knowledge of this value permits appropriate corrections and the relation between various measurable properties, and the cell volume, becomes possible. Trapped volume values reported in the literature, range from 0.5 to 10%, corresponding to various experimental systems, separation techniques and methods of measurement[1-5]. The rapid and rather accurate differential flotation method (DF)[1] is particularly useful when kinetics are studied with radioactive tracers[6].

In the present study, the volume trapped between red blood cells after DF separation was evaluated and compared with the volume trapped after ordinary centrifugation, using I^{131} serum albumin as tracer. The adsorption of I^{131}-labeled serum albumin, the transport of inorganic iodine across the cell membrane and hemolysis due to manipulation throughout the experiment, are accounted for.

Materials and methods. Blood was withdrawn from rabbits which received weekly injections of about 3 μCi of F_e^{59} per rabbit, over a period of 10 weeks. It was heparinized and diluted (1:1) with saline (0.16 *M* NaCl, 0.005 *M* KCl, 0.005 *M* phosphate buffer solution at pH 7.3).

Labeled human serum albumin (HSA) was added to that suspension to a final concentration of about 6 μCi/ml. The cells were then separated from the suspension medium and leucocytes by the DF method[1,7]. In this method cells are passed through phtalate ester solutions (Miles Yeda, Israel) of lower density than that of the cells. After centrifugation, a layer of water non-miscible phtalate solution separates the cells from the suspending medium. Hematocrites were obtained, and the relation between pellet volume and the radioactivity of F_e^{59} hemoglobin was established.

The DF separations were carried out in 0.4 ml polyethylene tubes. Each tube contained 0.05 ml of the separating fluid to which 0.1 ml of the suspension was added. Following centrifugation for 5 min at about 10,000 *g* the tubes were cut through the separating fluid, close above the pellet, with a razor blade. The radioactivity of the separated supernatant and pellet was then determined. An experiment in which erythrocytes were separated by ordinary centrifugation, was carried out for comparison purposes. A sample of blood diluted with saline was separated by the DF method, and another sample of the same blood was separated simultaneously, by ordinary centrifugation, in capillaries. After the separation, the capillaries were deep frozen for about 10 min and later cut with a glass knife through the pellet, so that contamination with the supernatant was avoided. Radioactivity of F_e^{59} hemoglobin served for evaluation of erythrocytes volume.

From radioactivity measurements of samples removed from the central portion of the separating fluid, it was found that the radioactivity transfer from the plasma-saline supernatant to the pellet, resulting from residual separating fluid, was less than 1/500% of the activity in the supernatant, and could be neglected.

Estimation of errors. Errors resulting from adsorbtion of I^{131}-HSA on the surface of the cells and possible transport of I^{131} through the cell membranes, were estimated as follows: Aliquots of packed cells, were added to (pipettes, previously wetted with saline were employed) plasmasaline solutions containing various concentrations of I^{131}-HSA: a) none, b) 0.04 μCi/ml, c) 0.15 μCi/ml. Samples of 0.1 ml were then separated by the DF method as above, the radioactivity of the pellets measured, and the changes (δ) in the I^{131}-HSA concentration of the plasma-saline solutions, following the removal of the cells, were determined.

From the dependence of (δ) on the original I^{131}-HSA concentration (C) of the plasma-saline solutions, (Figure 1A), that concentration for which $\delta \leq |\varepsilon|$ could be determined by interpolation. $|\varepsilon|$ is a number smaller than the value of the I^{131}-HSA concentration, expected from the dilution of the trapped volume by the plasma-saline solutions. When C is chosen so that $\delta \leq |\varepsilon|$, the I^{131} concentration of the pellet represents the contribution

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due to adsorption and transport of I^{131}. This value is used to correct the results obtained for the trapped volume. Let us describe the following example:

If there is neither adsorption nor transport in sample a), then δ_a results from the dilution of the volume trapped Δv_1 accompanying the packed cells in sample (a). In this case the radioactivity reaching the pellet after the second DF separation will correspond to $\Delta v_2/V_2$ and its radioactivity concentration will be the diluted value of the concentration which corresponds to $\Delta v_1/V_1$. In this case δ_a is larger than $|\varepsilon|$. If in another example, $\delta_a \simeq 0$, it can be deduced that the value obtained for the trapped volume after the first separation, $\Delta v_1/V_1$, originates exclusively from adsorption and penetration. In this case, the radioactivity obtained in the pellet after the second DF separation will be of the same concentration as that obtained after the first DF separation.

The reverse is not necessarily correct. It might happen that the value obtained for the trapped volume Δv_1 originates exclusively from adsorption and penetration and still one can get $\delta \neq 0$, since adsorption to the cell surface and penetration through the cell membrane depend on the concentration of the labeled serum albumin in the solution. $\delta \neq 0$ may thus result from release of adsorbed serum albumin molecules into a very dilute solution.

An additional check on the value of the correction due to adsorption and transport could be obtained from the dependence of the radioactivity per unit volume of the pellet (A_p) on that of the supernatant (A_s), after the second DF separation (Figure 1B).

Hemolysis was evaluated in order to correct the value for the total volume of cells in the sample. This was done by measuring the Fe^{59} in the supernatant and in the pellet.

In the case of complete hemolysis (ghosts), the red cell ghosts remain in the supernatant following centrifugation, when the density of the phtalate solution is higher than 1.02 g/cm³. In our experiments, phtalate fluids of densities greater than 1.062 g/cm³ were employed. Hemolysis was less than 5%, and the corresponding alternation (less than 5%) in the trapped volume value could be neglected to a first approximation.

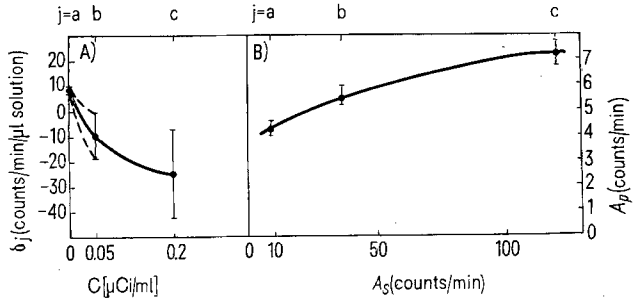

Fig. 1. A) The dependence of the change in the concentration of I^{131}-HSA in the plasma saline solutions, (δ), following the removal of cells, on the original I^{131}-HSA concentration (C) of the plasma saline solutions. The large errors in the δ values are not a matter of concern, as the error in the value of the correction will change the 'real' trapped volume by only about $\pm 1\%$ as a result of the uncertainty in the interpolated value, which corresponds to $\delta \simeq 0$ (see methods). This is relatively insignificant to the 10% accuracy of the experiment.

B) Correlation of the radioactivities per unit volume of the pellets (A_p) and supernatants (A_s) after the second DF separation. The slope of the curve in the interval b) to c) corresponds to the trapped volume following the second DF separation while the radioactivity A_p (see Figure A) around the region a) is due mainly to adsorption.

It can be supposed that partially damaged cells might reach the pellet, after having exchanged some of their hemoglobin with I^{131} serum albumin of the suspension. The correction corresponding to such an event is included in the correction for adsorption and transport. If this exchange has taken place, the trapped volume value of the 2 successive DF separations would be larger in the second separation than in the first. This was not found to be the case, and therefore it is reasonable to assume that either the partially damaged cells did not reach the pellet, or if they did, no appreciable exchange with serum albumin took place.

Radioactivity measurements. Radioactivity was determined by measuring the photopeaks of the γ radiation (1.3 MeV) for F_e^{59} and (0.36 MeV) for I^{131}, using a Na I well-type scintillation counter connected to a multichannel analyser. Most of the β-radiation was screened out.

Materials. Ferric ion in a soluble containing 1% sodium citrate, 10 mc/mg of Fe, was supplied by the Radiochemical Centre, England. Iodinated human serum albumin, 10 mg/ml, isotonic, sterile, pyrogen free solution, with about 0.1 iodine atom per protein molecule, was supplied by Sorin, Italy. It was dialysed against the NaCl phosphate buffer for 24 h at 4 °C.

Calculation procedure. The trapped volume (TV) is defined by $\Delta v/v$, where v is the total volume of the cells pellet, and Δv is the volume of the intercellular liquid in that pellet. Since the concentration of the labelet molecules in the intercellular space of the pellet and in the supernatant is the same, it follows that

$$\alpha' = \frac{A_p}{A_s} = \frac{\Delta v_p}{V_s}$$

Δv_p is the trapped volume in the pellet p, the radioactivity of which is A_p, corresponding to the volume v_p of the cells. V_s is the volume of the plasma-saline supernatant, the radioactivity of which is A_s. The trapped volume following the first separation of the cells, is therefore

$$\left(\frac{\Delta v}{v}\right)_1 = \frac{\Delta v_p}{v_p} = \frac{\alpha' V_s}{V_p - \alpha' V_s} = \frac{\alpha}{1-\alpha}$$

where $$\alpha = \frac{V_s}{V_p}\frac{A_p}{A_s}$$

and V_p is the total volume carried by the wet pipette from the pellet

$$V_p = v_p + \Delta v_p$$

For the second separation of cells, following the dilution of the packed cells in the plasma-saline solution, the procedure is as follows: If V_p is the total volume taken from the pellet obtained after the first separation, to be diluted, v_{p1} the total volume of cells transported, and Δv_{p1} the trapped volume calculated from the result obtained after the first separation, then $V_p = v_{p1} + \Delta v_{p1}$. The total volume V_{p2} of cells obtained in the second separation is then calculated from the known values of v_{p1}, Δv_{p1}, V_D and the dilution factor of the packed cells in solutions a, b, c, (see methods).

$$\alpha_2 = \frac{A_{p2}}{A_{p2} + A_{s2}} = \frac{\Delta v_{p2}}{V_D - v_{p2}}$$

where V_D is the volume of the diluted suspension of cells. This volume was 100 µl in the present experiments, so that, in units of µl:

$$\alpha_2 = \frac{\Delta v_{p2}}{100 - v_{p2}}$$

Trapped volume values in rabbit and mouse erythrocytes, after centrifugation

Experiment	Medium	Centrifugation	Correction due to adsorption and transport (%)	Trapped volume (corrected) $\Delta v/v \times 100$ [%]
Rabbit	Plasma-saline	10,000 g for 5 or 10 min	3–10	1.6 ± 0.2
		3000 g for 20 min		2.0 ± 0.2
		1000 g for 50 min		2.6 ± 0.3
	Saline	10,000 g for 5 min	40[a]	1.4 ± 0.3
	Plasma-saline	Centrifugation without phthalate solution 10,000 g for 15 min		1.6 ± 0.2
Mouse		10,000 g for 5 min	10	1.8 ± 0.2

[a] Since plasma proteins are not present in the suspending medium, high adsorption of I^{131}-HSA to the cells is observed. The max. values of cells hemolysis, after separation of erythrocytes by DF, were (1.1 ± 0.1)% and (3.6 ± 0.4)% for 1 and 2 separations, respectively.

and the trapped volume following the second separation is

$$\frac{\Delta v_{p_2}}{v_{p_2}} = \frac{\alpha_2\,(100\text{-}v_{p_2})}{v_{p_2}}$$

where $$\alpha_2 = \frac{A_{p_2}}{A_{p_2} + A_{s_2}}$$

The A's represent the radioactivity of the samples, and the indices '2' indicate that the samples were those obtained after the second DF separation.

Results. Trapped volume determinations were performed on various cell types, different media, and at different centrifugation speeds. The experiments with rabbits were performed on 3 animals. The experiment with mice was conducted with 2 animals which were injected i.v. with about 0.2 μCi of Fe^{59} twice, in 10 days interval. The results are summarized in the Table.

Discussion. The trapped volume value of 1.6% ± 0.2% obtained by DF at 10,000 *g* in this study is close to the value of 2% obtained by VAZQUEZ et al.[2] who separated the cells by simple centrifugation at about 2000 *g*, without correcting for adsorption or hemolysis. These authors found that the trapped volume was greater at the upper layers of the pellet, 2.6% in the upper half, and 2% in the lower. Discrepancies with the results of the present study are probably due to the lower centrifugation speed used by these authors, which may also account for the incomplete sedimentation at the top of the tubes. The 2% trapped volume obtained in this work, after centrifugation at 3000 *g*, and 2.6% at ~1000 *g* are close to their results. The 5% trapped volume obtained by BALLENTINE and BURFORD,[1] who separated (*Tetrahymena*) by the DF method at about 2000 *g*, is probably associated with surface characteristics of these cells and eventual pinocytosis of the labeled molecules. The nature of the suspension medium might have also contributed to the relatively large trapped volume. The low value of 0.5% obtained by KLIENE and BURGASS[3] results from their linear extrapolation of the function which correlates electrical conductivity and trapped volume in the low conductivity region which is questionable[5]. Under the same experimental conditions, and with the same membrane adsorption properties, assuming that the cells are deformable, the trapped volume is proportional to the surface to volume ratio of the cells. The 10% trapped volume reported for *E. coli*[4] and the value of 11% obtained by the author for *B. megaterium*[6], are therefore in agreements with the value for erythrocytes reported in the present study.

The larger trapped volume value obtained, in this work, for smaller centrifugational fields, suggests that some residual intercellular liquid escapes from the pellet into the supernatant, when larger centrifugational fields are employed.

A trapped volume of 1.6% corresponds to an average intercellular distance of about 150 Å, when the cell volume is taken as 100 μm^3, assuming random orientation of the cells in the pellet. If the orientation of the cells is ordered rather than random, or the cells are spherical, of very similar diameter and not deformable, the spaces between the cells, even in the most compact configuration would exceed the trapped volume of the normal deformable bidiscoidal cells. Water which might flow from the cells into the intercellular spaces of the pellet (when the cells are not deformable enough) will not appear in the present measurements, which represent the trapped volume originating from the experimental medium.

Zusammenfassung. Das Zwischenzell-Volumen an Erythrozyten wurde mit 1.6 ± 0.2% bestimmt, was bei einem mittleren Zellvolumen von 100 μm^3 einer durchschnittlichen Interzellulärdistanz von 150 Å bei zufälliger Orientierung der Zellen entspricht.

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