Active Transport and Enzymes of the Erythrocyte Membrane under Protein Deprivation

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(Received 4 March 1965)

1. Starvation for 3 days causes membrane damage of the rat erythrocyte manifested by several alterations. The adenosine-triphosphatase activity is decreased but that of acetylcholinesterase is not affected. 2. The ouabain-sensitive adenosine-triphosphatase activity increases at the expense of the non-sensitive enzyme moiety. 3. The Rb^+ uptake is not altered but the galactose transport is accelerated by the stated experimental conditions. 4. The modifications induced by starvation do not recover on re-feeding.

Starvation and a protein-free diet produced a decrease of several enzyme activities involved in the oxidation-reduction reactions of erythrocyte metabolism (Sánchez de Jiménez, Torres, Valles, Solís & Soberón, 1965). The enzymes affected are found in the cytoplasm of the cell and their changes were interpreted as a consequence of a more rapid breakdown. Because it is known that the properties of enzymes vary according to whether they are in solution or integrated in particles (Green, 1957), it seems likely that the enzymes may be better preserved when they are linked to structures like the cell membrane. Accordingly, it was decided to explore the behaviour of enzymes located in the stroma and some of the functional characteristics of the membranes of erythrocytes obtained from animals submitted to starvation.

The erythrocyte has been extensively used as a model to study the mechanism of active transport through membranes. It has been established by several authors that ATPase* (ATP phosphohydrolase, EC 3.6.1.4) is an anisotropic enzyme that plays a fundamental role in this function (for reviews see Hokin & Hokin, 1963; Judah & Ahmed, 1964). It is located in the cell membrane and selectively stimulated by intracellular Na^+ and extracellular K^+ (Whittam, 1962). Acetylcholinesterase (EC 3.1.1.7) is another stromal enzyme, but this is not related to active transport (Mathias & Sheppard, 1954). It was considered that the study of these enzymes as well as the transport through the erythrocyte membrane may reflect any damage induced in this structure by starvation.

The behaviour of ATPase and acetylcholinesterase together with the assessment of the capacity

of the cells in active (ions) and non-active (galactose) transport processes has been investigated in the erythrocytes of normal and nutritionally deficient animals.

MATERIALS AND METHODS

Normal male Wistar rats weighing 180–200 g., fed with a well-balanced commercial diet (Purina Chow), were used as control (group A). Another group of rats of similar weight were starved for 3 days (group B). The third group (C) was formed by animals starved for 3 days and re-fed with the normal diet for 15 days. The removal of blood and the processing of the samples (in individual determinations or by pooling equal samples obtained from animals of a given group) were carried out as described by Sánchez de Jiménez *et al.* (1965). The protein concentration was measured by the procedure of Lowry, Rosebrough, Farr & Randall (1951). Lipids were measured gravimetrically after Soxhlet extraction. Dry weight was determined by heating at 100° a sample of washed packed erythrocytes to constant weight.

Acetylcholinesterase was determined by following the disappearance of acetylcholine (Robbins, Hopkins & Roth, 1958) in haemolysates containing the fragmented membranes. ATPase was assayed by measuring the inorganic phosphate released from ATP; the incubation system was set up as indicated by Caffrey, Tremblay, Gabrio & Huenekens (1956) but the concentrations of ionic species were followed by the method recommended by Post, Merritt, Kinsolving & Albright (1960) in the presence and in the absence of ouabain. The assay of ATPase was carried out in the same type of haemolysate as above, and also in intact cells (Whittam, 1958). The concentrations of the ions employed are given in each experiment.

One unit of enzyme activity is defined as the amount of enzyme required to convert 1 μmole of substrate into product/min.

For the active-transport experiments, the uptake of $^{86}\text{Rb}^+$ was tested in the presence and in the absence of ouabain as described by Hashimoto & Yoshikawa (1963),

* Abbreviation: ATPase, adenosine triphosphatase.

except that intact erythrocytes were utilized and the radioactivity was assayed in the cells. For this purpose a well scintillator device was used. The $^{86}\text{RbCl}$ was purchased from Abbott Laboratories (Chicago, Ill., U.S.A.) with a specific radioactivity of 10.18 mc/mg. For the galactose experiments the uptake was determined under the conditions described by Lacko, Burger, Hejmová & Rejnková (1960).

RESULTS

Experiments were carried out to determine optimum conditions to ensure initial-velocity measurements of the enzymes investigated. The systems were set up with optimum concentrations of substrates and ionic species so that the amount of enzyme was limiting. The optimum pH was found to be 7.3 for ATPase and 7.0–8.2 for acetylcholinesterase. It was also found that ATPase and acetylcholinesterase are relatively stable when stored at 4° (85% of the initial activity remains after 2 and 3 days respectively); they are also resistant to heat denaturation (60° during 30 min.

for acetylcholinesterase and 55° during 10 min. for ATPase are necessary to show the first indication of enzyme damage).

The results in Table 1 show the effect of starvation on the membrane enzymes being studied; the assays were carried out on haemolysates containing the fragmented membranes. ATPase activity is significantly diminished ($P < 0.01$ in comparing groups A and B; $P < 0.05$ in comparing groups A and C), whereas acetylcholinesterase activity remains practically unchanged.

The lipid content of the erythrocyte, which is mainly located on the membrane, and the solid material of the cell do not appear to be altered by the experimental conditions employed (Table 2).

Because it is well established that some of the ATPase activity, i.e. that inhibited by ouabain, participates in active transport, the enzyme rate and the Rb^+ uptake were investigated. The experiments were performed on intact cells in the presence and in the absence of ouabain. ATP was added to

Table 1. *Effects of starvation on the ATPase and acetylcholinesterase activities of the rat erythrocyte*

The numbers of animals used in individual determinations as well as those utilized for the 'pooling' procedure (Sánchez de Jiménez *et al.* 1965) are indicated; for the latter, the numbers of pooling experiments are given in parentheses. For the calculation of the statistical significance the results obtained by each experiment following the latter method were considered as one individual value. They are expressed as means $\pm$ s.e.m. The difference in ATPase activity between the normal group and each of the two other experimental groups is significant ($P < 0.01$). The incubation system for the assay of ATPase was set up as follows: 0.5 ml. of haemolysate (1 ml. of packed cells to 4 ml. of water) containing fragmented membranes, 500 μ moles of glycine buffer, pH 7.5, 25 μ moles of MgCl_2 , 5 μ moles of KCl, 5 μ moles of NaCl and 25 μ moles of ATP, in a final volume of 3.0 ml. The incubation system for acetylcholinesterase was set up as follows: 0.5 ml. of haemolysate as above, 200 μ moles of phosphate buffer, pH 7.5, and 8 μ moles of acetylcholine, in a final volume of 1.5 ml. The substrate was kept as a 40 mM solution in Imm-acetate buffer, pH 4.5.

	No. of animals		10 ³ × ATPase activity (unit/mg. of protein)	No. of animals		10 ³ × Acetylcholinesterase activity (units/mg. of protein)
	Individual determinations	'Pooling' procedure		Individual determinations	'Pooling' procedure	
Normal	7	(6)	0.4631 $\pm$ 0.0193	26	(3)	2.24 $\pm$ 0.05
Starvation	5	(3)	0.3897 $\pm$ 0.0110	13		2.47 $\pm$ 0.20
Recovery from starvation	4	(6)	0.3714 $\pm$ 0.0352	3 8	(1) (6)	2.50 $\pm$ 0.13

Table 2. *Effects of starvation on dry weight and lipid content of the rat erythrocyte*

The number of animals is indicated as explained in Table 1. The results for dry weight are expressed as the values obtained in each pooling experiment performed and for lipid as means of individual determinations $\pm$ s.e.m.

	No. of animals	Dry wt. (mg./ml. of erythrocytes)	No. of animals	Lipid content (mg./ml. of erythrocytes)
Normal	16 (3)	248.1	24	96.12 $\pm$ 3.74
Starvation	16 (3)	221.3	26	95.45 $\pm$ 5.78
Recovery from starvation	16 (3)	231.9	10	108.39 $\pm$ 8.63

the incubation medium of the whole erythrocyte because, even though it has been reported that the anisotropic ATPase utilizes the ATP from the interior of erythrocyte 'ghosts' (Whittam, 1962), we found that the addition of ATP to the incubation system containing erythrocytes from normal or starved animals produced an increment of the rate

Fig. 1. Rate of ATP hydrolysis by whole erythrocytes in the presence and in the absence of added ATP. The incubation system contained 25 μ moles of $MgCl_2$, 30 μ moles of KCl, 0.12 ml. of packed cells and 900 μ moles of glycine-HCl buffer, pH 7.5, in a final volume of 3.0 ml. ○, 25 μ moles of ATP added; ●, ATP omitted.

as well as a prolonged time during which linearity of hydrolysis is observed (Fig. 1). Further, when ATPase was measured as a function of time, a plateau was reached and a new addition of ATP could enhance the rate again. It was made certain that ATPase was measured under conditions of initial velocity, where the enzyme was the only limiting factor. Moreover, the fact that the percentage decreases in the ATPase activity caused by starvation are similar, whether determined in whole cells or in haemolysates containing fragmented membranes (Table 1), is further support for choosing the experimental conditions of the assay as indicated.

The results presented in Table 3 show, besides the decrease of ATPase activity noted above, that ouabain inhibited the enzyme activity of the erythrocytes from starved animals to a greater extent than that of the normal cells (22.9, 44.2 and 38.5% for groups A, B and C respectively). It also gives the values for the Rb^+ uptake, which seemed to be unaffected by starvation. The cells incubated in the presence of ouabain showed some radioactivity that is difficult to interpret as indicative of active transport because the concentration of the inhibitor in the medium was 100 times the value of its K_i (Post *et al.* 1960; Dunham & Glynn, 1961).

To be sure that the uptake of Rb^+ at 90 min. did not hide differences between the experimental groups, the velocity of entrance of the ion to the cells was recorded (Fig. 2). In the absence of the glycoside the rates of Rb^+ uptake are very similar for groups A, B and C and a definite tendency to reach a plateau is observed. The curves obtained in the presence of ouabain do not start at the origin and are clearly of a different trend, which suggests

Table 3. Effects of ouabain on ATPase activity and Rb^+ uptake of intact erythrocytes obtained from normal and starved animals

The enzyme activity and the active transport are expressed as units and counts/min./ml. of packed erythrocytes respectively. Experimental groups: A, normal; B, starved for 3 days; C, starved for 3 days and re-fed with the normal diet for 15 days. The incubation system for the ATPase assay was set up as follows: 25 μ moles of $MgCl_2$, 30 μ moles of KCl, 25 μ moles of ATP, 0.12 ml. of packed cells and 900 μ moles of glycine-HCl buffer, pH 7.5, in a final volume of 3.0 ml. The incubation system for the assay of Rb^+ uptake was as follows: 2.76 μ moles of $MgCl_2$, 0.075 μ mole of $^{86}RbCl$ (specific activity 10.18 mc/mg.), 125 μ moles of sucrose, 20 μ moles of ATP and 0.3 ml. of packed cells, in a final volume of 0.33 ml.

Experimental group	ATPase activity (units/ml. of packed erythrocytes)		$^{86}Rb^+$ uptake (counts/min./ml. of packed erythrocytes)	
	Ouabain absent	With 3.3 mM-ouabain	Ouabain absent	With 3.3 mM-ouabain
A	2.09 $\pm$ 0.07	1.57 $\pm$ 0.11	7077 $\pm$ 448	2004 $\pm$ 387
B	1.83 $\pm$ 0.04	1.07 $\pm$ 0.02	7180 $\pm$ 614	1820 $\pm$ 77
C	1.60 $\pm$ 0.06	0.98 $\pm$ 0.06	7173 $\pm$ 434	1900 $\pm$ 421

Fig. 2. Rate of $^{86}\text{Rb}^+$ uptake by erythrocytes obtained from normal and starved animals. The intact erythrocytes were incubated under the conditions indicated on Table 3 in the absence ($\circ$, $\triangle$, $\square$) and in the presence ($\bullet$, $\blacktriangle$, $\blacksquare$) of ouabain. At the indicated times the cells were spun down and washed three times with 0.9% NaCl, and the radioactivity was assayed in a well scintillator. Experimental groups: $\circ$ and $\bullet$, normal; $\triangle$ and $\blacktriangle$, starved for 3 days; $\square$ and $\blacksquare$, starved for 3 days and re-fed for 15 days.

Table 4. *Effect of starvation on non-active transport by the rat erythrocyte*

To 0.2 ml. of packed erythrocytes was added 10 ml. of a 0.5% (w/v) galactose solution and the mixture was incubated for 90 min. at 37° . The sugar uptake was measured by the Somogyi method (Nelson, 1944). The determinations were carried out on individual animals and the results are expressed as means $\pm$ s.e.m. The significance test was applied to the difference between the normal group and each of the two experimental groups.

	No. of animals	Galactose uptake ($\mu\text{g.}/\text{ml.}$ of erythrocytes)	Significance
Normal	26	389 ± 30.6	
Starvation	20	476 ± 13.4	$P < 0.05$
Recovery from starvation	23	519 ± 29.3	$P < 0.01$

that the radioactivity assessed in the cells after incubation and centrifugation might be due to adsorption of the radioactive ion on their surface.

The experiments performed on galactose trans-

port are presented in Table 4. This sugar goes through the membrane by a non-active mechanism defined as facilitated transport (Widdas, 1954). There is a remarkable increase in the galactose uptake induced by starvation.

DISCUSSION

It is difficult to imply from the results obtained in the present work that linkage of proteins to structures render them less susceptible to the deleterious effect of starvation. Indeed, although ATPase activity was significantly lessened, acetylcholinesterase activity was not affected. Concomitantly with the diminution of total ATPase activity there was an augmentation of that portion of the enzyme protein which is sensitive to ouabain. The erythrocytes from starved animals had approx. 0.74 unit of ouabain-sensitive ATPase as compared with 0.51 unit in the normal erythrocytes. This suggests that wherever changes are introduced by food deprivation they are able to convert the ouabain-insensitive enzyme molecules into species susceptible to its inhibitory effect. That such might be the case is further supported by the fact that the aging of erythrocyte 'ghosts' produces a similar effect (Hokin & Reasa, 1964), by the observation that deoxycholate added to brain microsomes increases the Na^+ - K^+ -sensitive ATPase at the expense of the non-sensitive enzyme moiety (Järnefelt, 1964), and also by the work of Askari & Fratantoni (1964), who concluded that the different responsiveness of erythrocytic ATPase to Na^+ and K^+ did not correspond to more than one enzyme.

Although Nakao, Nagano, Adachi & Nakao (1963) were able to extract two different fractions of ATPase activity from erythrocyte membranes by means of different concentrations of sodium iodide, it is likely that the solubility properties manifested by the two enzyme fractions may be a consequence of its association with other components in the membrane structure, susceptible to be modified either by starvation (this paper), detergents (Järnefelt, 1964), ultrasonic treatment (Askari & Fratantoni, 1964) or aging (Hokin & Reasa, 1964).

It has been demonstrated that Rb^+ and K^+ cause a similar stimulation of the erythrocyte ATPase (Whittam & Ager, 1964); thus the use of the former in transport studies may reflect the physiological handling of K^+ . The rate of Rb^+ uptake is not greater in the erythrocytes from starved animals.

It was also corroborated in the present work that the alterations induced by starvation are not reversed by re-feeding of the animals (Sánchez de Jiménez *et al.* 1965). In fact, the galactose transport seemed to be more greatly disturbed in the cells of the animals of group C than in the cells of the

animals of group B. This, as advanced by Sánchez de Jiménez *et al.* (1965), is in accordance with the lack of biosynthetic pathways in the erythrocyte.

This work was supported by a U.S. Public Health Service grant (AM05766) from the National Institutes of Health. Thanks are due to Dr R. Whittam for his valuable suggestions and his interest in this work. The able technical assistance of Miss M. Eugenia Fonseca is fully recognized.

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