Studies on the Oxidation-Reduction Systems of the Erythrocyte

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1. Starvation for 3 days produces a decrease in methaemoglobin-reductase and glutathione-reductase activities, but it does not alter the glucose 6-phosphate-dehydrogenase activity of the rat erythrocyte. 2. The feeding of a protein-free diet for 11 days causes greater changes in the first two enzymes and also a diminution of the third. Under this experimental condition slight decreases in protein and haemoglobin contents were noted. 3. The experimental animals did not show methaemoglobinaemia, probably because the activity of methaemoglobin diaphorase is preserved. 4. The GSH content was not affected but the stability of the tripeptide in the presence of an oxidizing agent was diminished.

It is well known that starvation and protein-deficient diets affect different tissues in varying degrees; whereas great changes are produced in liver (Soberón & Sánchez, 1961), brain is less influenced (Wainio, Allison, Kremzner, Bernstein & Aronoff, 1959). Low protein ingestion affects the amount of haemoglobin present in the erythrocyte (Metcoff, Favour & Stave, 1945). It was decided to explore the behaviour of the erythrocyte, since it has a definite life-span (Allison, 1960), it is unable to carry out protein and lipid synthesis (London, 1960), and it is endowed with a group of enzymes directed towards the maintenance of haemoglobin in the reduced state (Huennekens, Caffrey, Basford & Gabrio, 1957). The enzymes involved in the preservation of haemoglobin in the reduced state were chosen for the present study, as it was expected that they would provide indications as to the effect of the deficiencies on the metabolic pattern of the erythrocyte.

The present paper is concerned with the changes observed in the activities of methaemoglobin reductase, methaemoglobin diaphorase, glutathione reductase (EC 1.6.4.2) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49), as well as variations in the GSH and nicotinamide nucleotide contents observed in the erythrocytes of rats submitted either to starvation or to protein deprivation.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 180–190 g. (mean 187 g.) were divided in four groups. Group A was fed with an adequate commercial diet (Purina Chow), group B was fed with a protein-free diet (Flores, Rosado, Torres & Soberón, 1962), group C was starved for 3 days, and group

D was re-fed for 15 days with the normal diet after starvation for 3 days.

Preparation of samples. Blood for analysis was taken only once from each animal by cardiac puncture after ether anaesthesia, with heparin as anticoagulant. The samples were processed individually or after pooling several of them pertaining to a given experimental group; for this purpose samples of equal size were mixed. The cells were centrifuged at 0° at 1600g for 10 min. and washed three times with 0.9% NaCl. The contamination by white cells was determined in every case and it consistently corresponded to 1:10000. A 7 ml. sample of the packed cells was made up to 10 ml. with 0.9% NaCl solution in a graduated centrifuge tube, and from this suspension haemolysates of adequate concentrations were used for the different determinations.

There was no significant difference in the results obtained by processing the samples by any of the two stated procedures (Table 1).

Enzyme assays. It was originally planned to explore the enzyme activities connected with the NADP⁺-NADPH oxidation-reduction system (methaemoglobin reductase, glucose 6-phosphate dehydrogenase and glutathione reductase); nevertheless, for reasons explained below, methaemoglobin diaphorase was also studied.

Glucose 6-phosphate dehydrogenase was determined by following the NADP⁺ reduction at 340 m μ (Kornberg & Horecker, 1955); the incubation system contained 1000 μ -moles of tris-HCl buffer, pH 7.5, 10 μ -moles of MgCl₂, 1.0 μ -mole of NADP⁺, 10 μ -moles of glucose 6-phosphate and 100 μ l. of haemolysate in a final volume of 3.0 ml.

Methaemoglobin reductase was measured by the change in extinction at 340 m μ produced by the oxidation of NADPH (Huennekens *et al.* 1957) in a system containing 270 μ -moles of methylene blue, 600 μ -moles of NADPH, 100 μ l. of haemolysate and 255 μ -moles of phosphate buffer, pH 7.5, in a final volume of 3.0 ml.

Glutathione reductase was determined by following the oxidation of NADPH at 340 m μ (Racker, 1955), in a system containing 270 μ -moles of phosphate buffer, pH 7.5, 2 μ -moles

of GSSG, 600 μ moles of NADPH and 100 μ l. of haemolysate in a final volume of 3.0 ml.

For glucose 6-phosphate dehydrogenase, methaemoglobin reductase and glutathione reductase, 1 unit of enzyme activity is defined as the amount of enzyme that reduces or oxidizes 1 μ mole of the cofactor/min. under the stated conditions. For methaemoglobin diaphorase 1 unit is defined as the amount of enzyme that produces a change of 0.100 in extinction/min. under the assay conditions.

Kinetic studies were carried out to determine optimum conditions for the assay of the enzymes to ensure initial velocities, good sensitivity and reproducibility. Some of the results obtained with these studies for the NADP-dependent enzymes investigated are shown in Table 2.

Methaemoglobin diaphorase was assayed by the diminution in extinction at 600 $m\mu$ (Ross, 1963) in a system containing 50 μ moles of tris-HCl buffer, pH 7.5, 1.0 μ mole of

EDTA, 12 μ moles of 2,6-dichlorophenol-indophenol, 17.6 μ moles of NADH and 300 μ l. of a haemolysate prepared as recommended by Ross (1963) in a final volume of 3.0 ml.

Cofactors and parameters of reference. GSH was measured by forming the addition compound with alloxan (Patterson & Lazarow, 1955). GSH stability was determined by the method described by Beutler, Dern, Flanagan & Alving (1949). Oxidized nicotinamide nucleotides were assayed as described by Lowry, Roberts & Kappahn (1956). Protein was measured by the procedure reported by Lowry, Rosebrough, Farr & Randall (1951). Erythrocyte mass was measured by following the incorporation of ^{59}Fe (Gibson, 1946): 1.5–2.0 μC of $^{59}\text{FeSO}_4$ was injected into a donor rat by the tail vein; 24 hr. later 3 ml. of blood was drawn by cardiac puncture, the erythrocytes were separated and washed and the radioactivity was determined in a sample by haemolysing the erythrocytes with saponin (approx. 200 mg. for the erythrocytes corresponding to 1 ml. of blood) and assayed in a well scintillation counter. The labelled erythrocytes originally contained in 1 ml. of blood were perfused by the tail vein in each of the experimental rats; 24 hr. later blood was removed and the radioactivity measured as indicated. Haemoglobin and haematocrit measurements were carried out by conventional methods.

RESULTS

The effects of the experimental conditions employed on several biological parameters of the rat erythrocyte are shown in Table 3. Though the erythrocyte mass was not greatly affected, there was a tendency for the haematocrit to increase. The protein and haemoglobin contents were not altered by starvation and only slightly diminished in the animals fed with a protein-free diet. The presence of activators or inhibitors was not disclosed by the enzyme–time relationship test carried out in each case.

Table 4 presents the effects of the protein-free diet and starvation on the NADP-dependent enzyme activities. Both experimental conditions greatly decreased methaemoglobin-reductase and glutathione-reductase activities, the latter to such

Table 1. *Comparison of the activities of NADP-dependent enzymes of rat erythrocytes obtained with the two procedures employed for processing the samples*

The individual determinations were carried out in separate samples obtained from ten animals, and the results are expressed as means $\pm$ S.E.M. For the 'pooling' procedure equal portions from 15–20 animals were mixed and processed, and the values given correspond to different experiments. One unit of enzyme activity is defined as the amount of enzyme that reduces or oxidizes 1 μ mole of the cofactor/min. under the assay conditions.

Enzyme	$10^3 \times$ Enzyme activity (units/mg. of protein)	
	Individual determinations	'Pooling' procedure
Glucose 6-phosphate dehydrogenase	2.8 ± 0.052	2.4 2.8
Methaemoglobin reductase	1.17 ± 0.09	1.1 1.4 1.5
Glutathione reductase	0.613 ± 0.036	0.7 0.7

Table 2. *Apparent K_m , optimum pH and energy of activation of NADP-dependent enzymes of the rat erythrocyte*

The incubation conditions of the systems are given in the text.

Enzyme	Apparent K_m	Optimum pH	Energy of activation (cal./mole)
Glucose 6-phosphate dehydrogenase	77.5–90 μM for glucose 6-phosphate (at 500 μM of NADP $^+$); 120 μM for NADP $^+$ (at 5 μM of glucose 6-phosphate)	7.25–7.60	9000
Methaemoglobin reductase	6–10 μM for NADPH	7.5	10000
Glutathione reductase	42–43.3 mM for GSSG (at 0.30 μM of NADP $^+$)	7.3–7.6	9160

Table 3. *Effect of starvation and protein-free diet on different biological parameters of the rat erythrocyte*

For the determinations of body wt. the results are expressed as means $\pm$ s.e.m. of the percentage changes. For the other parameters the results are given as means of three different experiments (15–20 animals each) with the pooling procedure. The ranges are indicated in parentheses.

	Normal	Protein-free diet	Starvation	Recovery from starvation
Percentage change in body wt.	0	-18.9 ± 0.578	-14.7 ± 0.61	$+21.3^* \pm 1.25$
Erythrocyte mass (g./100g. body wt.)	3.13 (2.90–3.25)	3.62 (3.48–3.76)	3.09 (2.96–3.23)	—
Haematocrit	44.3 (43–45)	48.5 (46–49.5)	49.9 (48.6–55.8)	46.6 (45.5–47.5)
Protein†	170 (155–189)	148 (136–167)	169 (155–199)	178 (166–198)
Haemoglobin†	158 (152–168)	133 (127–139)	156 (150–165)	142 (136–150)

* Increment over the weight reached after 3 days of starvation.

† The values are expressed as mg. of material corresponding to the erythrocytes contained in 1ml. of blood.

Table 4. *Effects of starvation and protein-free diet on the NADP-dependent enzymes of the rat erythrocyte*

One unit of enzyme activity is defined as the amount of enzyme that reduces or oxidizes 1μ mole of the cofactor/min. under the conditions of each test. The experimental groups are as follows: A, normal rats; B, rats fed with a protein-free diet for 11 days; C, rats starved for 3 days; D, rats starved for 3 days and re-fed with the normal diet for 15 days. The results indicate enzyme units/mg. of protein (means $\pm$ s.e.m.). All enzyme activities, with the exception of glucose 6-phosphate dehydrogenase in group C, are significantly different from the normal value ($P < 0.001$). Glutathione-reductase activity in group B was not detected with the procedure employed.

$10^3 \times$ Enzyme activity (units/mg. of protein)

Group of animals	Glucose 6-phosphate dehydrogenase	Methaemoglobin reductase	Glutathione reductase
A	2.96 ± 0.008	1.016 ± 0.056	0.066 ± 0.0035
B	2.39 ± 0.007	0.310 ± 0.015	Not measurable
C	3.29 ± 0.011	0.706 ± 0.067	0.021 ± 0.0030
D	—	0.760 ± 0.062	0.029 ± 0.0032

an extent that it could not be detected with the procedure employed in one of the experimental groups. On the other hand, glucose 6-phosphate-dehydrogenase activity was less influenced. Protein deprivation produced in all cases more pronounced effects than starvation; also, re-feeding with the normal diet after the period of starvation did not achieve recovery of the damage caused.

The concentration of oxidized nicotinamide nucleotides increased as a result of the experimental conditions (Table 5). Because, in all likelihood, there is no exchange of these compounds with the environment and the mature erythrocyte is incapable of synthesizing them (London, 1961), the elevation should be produced at the expense of a fall in the concentration of the reduced nucleotides. Although the GSH concentration appears to be

Table 5. *Effects of protein-free diet and starvation on the oxidized nicotinamide nucleotide and GSH contents of the rat erythrocyte*

The experimental groups were as indicated in Table 4. The oxidized nicotinamide nucleotide contents of groups B, C and D are significantly increased with respect to the normal group ($P < 0.001$, $P < 0.001$ and $P < 0.001$ respectively). The apparent increase in GSH content of group B is due to a decrease in the protein concentration. The results indicate μ g./mg. of protein (means $\pm$ s.e.m.).

Group of animals	$10^2 \times$ Concn. (μ g./mg. of protein)	
	NAD ⁺ + NADP ⁺	GSH
A	28.25 ± 1.40	0.144 ± 0.008
B	53.50 ± 2.95	0.175 ± 0.012
C	43.25 ± 1.88	0.150 ± 0.008
D	41.00 ± 1.76	0.130 ± 0.007

Table 6. *Effect of starvation on the methaemoglobin-diaphorase activity of the rat erythrocyte*

The results are expressed as means $\pm$ S.E.M. One unit of enzyme activity corresponds to the amount of enzyme that produces a change in extinction of 0.1/min. under the assay conditions.

	No. of animals	$10^3 \times$ Enzyme activity (unit/mg. of protein)
Normal rats	14	0.323 ± 0.058
Rats starved for 3 days	13	0.467 ± 0.074
Rats starved for 3 days and re-fed with normal diet for 15 days	18	0.435 ± 0.108

elevated as a consequence of the protein-free diet, the change is probably determined by the decrease in protein content, since the values shown in Table 5 are expressed with reference to the amount of protein.

Although methaemoglobin-reductase activity was altered, the animals studied did not show any sign of accumulation of oxidized haemoprotein; this called for an investigation of methaemoglobin diaphorase, the other enzyme system capable of catalysing the same reaction which is NAD-dependent (Ross, 1963). The results in Table 6 show that methaemoglobin-diaphorase activity is not changed significantly by the experimental conditions employed.

Since the GSH concentration was not significantly modified, even though glutathione-reductase activity was greatly impaired, the capacity of the cells to preserve the reduced GSH form was explored. When the erythrocytes from normal rats were incubated during 120 min. in the presence of acetylphenylhydrazine (Beutler *et al.* 1949), the GSH content fell from 240 ± 12 (S.E.M.) to 128 ± 10 $\mu\text{g./mg.}$ of protein, whereas the erythrocytes from animals starved for 3 days decreased their GSH content from 211 ± 28 to 75 ± 5 $\mu\text{g./mg.}$ of protein under the same conditions. These results clearly show that the erythrocytes with decreased glutathione-reductase activity are more susceptible to the addition of oxidizing reagents.

DISCUSSION

In the starved animals, as well as in those submitted to protein deficiency, the erythrocytes are not destroyed at the same rate as other tissues. This is inferred from the observation that in both catabolic conditions the erythrocyte mass is maintained in spite of the loss of body weight. Under the same circumstances liver, for instance, is greatly affected (Soberón & Sánchez, 1961).

There is no question, however, that important changes are caused in the erythrocyte by the influ-

ence of the experimental conditions employed in this work. The haemoglobin and protein contents did not vary as a result of starvation, yet they were slightly, but significantly, diminished by protein deprivation. Some of the enzymes investigated, namely methaemoglobin reductase and glutathione reductase, reflected more considerably the damage caused. It is clear that the lack of protein in the diet caused more important changes than starvation, which could be due to the longer experimental period (11 and 3 days respectively). Moreover, the decrease in enzyme activities observed in the first condition should be even more pronounced, since they are expressed with reference to the amount of protein.

It could be assumed that the life-span of the rat erythrocyte (50–60 days; Allison, 1960) is not lowered by the experimental conditions employed because such a change causes anaemia (Berlin, Waldmann & Weissman, 1959), which was not a significant feature of our experimental animals. If this is the case, it means that the variations observed were produced mainly in the existing erythrocyte population, since the magnitude of the changes is greater than the one that would be introduced if all new erythrocytes lacked a given constituent. This point is illustrated by the low activities of glutathione reductase and methaemoglobin reductase found in both experimental groups (Table 4). It appears then that the enzymes in the erythrocytes of the animals in groups B and C are broken down more rapidly than those of the normal animals. The enzymes investigated in the present work are not linked to any cellular structure and, in contrast, enzymes located in the stroma are more affected by the same experimental conditions (Sánchez de Jiménez, Valles, de León & Soberón, 1965).

That the damage caused by starvation is not restored by re-feeding of the animals is in accordance with the lack of biosynthetic capacity of the mature erythrocyte (London, 1960).

The present results throw light on the oxidation-reduction processes of the erythrocyte, which are illustrated in Scheme 1. It seems certain that the conversion of methaemoglobin into haemoglobin is mainly carried out by methaemoglobin diaphorase. Relevant to this are the studies that report the relative absence of this enzyme in cases of human congenital methaemoglobinaemia (Scott & Griffith, 1959; Harris, 1963). In addition, the maintenance of the GSH content, which is critical for the integrity of the cell (Fegler, 1952), seems to be achieved by the synthesis of the tripeptide, a process known to occur in the mature erythrocyte (Kasbekar & Sreenivasan, 1959). The erythrocyte is unable to exchange GSH with the environment (Spear & Sass, 1964).

Scheme 1. Oxidation-reduction systems of the erythrocyte. 1, Methaemoglobin diaphorase; 2, methaemoglobin reductase; 3, glutathione reductase; 4, glucose 6-phosphate dehydrogenase. G6P, Glucose 6-phosphate; 6PG, 6-phosphogluconate; Hb, haemoglobin; MetHb, methaemoglobin; other abbreviations are conventional.

The present findings reveal an interesting possibility that the effects of a protein-free diet can be detected with great sensitivity by assaying for specific enzymic activities. Therefore, since the erythrocytes are readily available, they may be suited to the study of biological parameters related to the nutritional status.

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