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Ethacrynic acid and iron uptake in the rabbit reticulocyte

The reticulocyte, induced either by bleeding or phenylhydrazine, has been studied widely for its iron-uptake properties. In contrast to the erythrocyte the reticulocyte takes up iron bound to transferrin, releases the metal from transferrin and moves iron into the cell by a process that is not yet clear. It seems that the transferrin–iron is attached to a membrane receptor in a union which shows metabolic dependence[1]. Recent work indicates that the transferrin molecule may pass into the cell and not be exclusively attached to the membrane[2]. The kinetics of interaction between transferrin–iron and the reticulocyte has been shown to resemble an antigen–antibody reaction[3].

MORGAN AND BAKER[4] have followed the uptake of transferrin and iron by rabbit reticulocytes after application of several types of inhibitors. Both transferrin and iron uptake were inhibited similarly by sulfhydryl reagents which in some cases could be prevented by prior addition of cysteine. Compounds, such as rotenone, inhibited iron uptake preferentially to transferrin uptake. The authors concluded that iron uptake by the reticulocyte is not simply dependent on respiration for a supply of ATP but that it is linked to the mitochondrial electron-transfer chain. Contrary to our results in which a mild inhibitory effect on iron uptake was obtained with ouabain[5], MORGAN AND BAKER[4] reported that the compound had no effect on either transferrin or iron uptake in the rabbit reticulocyte.

A second Na^+ pump which was thought unlikely to use ATP as an energy source, was postulated by HOFFMAN AND KREGENOW[6]. It is inhibited by ethacrynic acid but not by ouabain and seems to require Na^+ in the extracellular environment. More recently GORDON AND DE HARTOG[7], in studies with Ehrlich ascites tumor cells and human red blood cells, have obtained evidence that ethacrynic acid inhibits ATP generation from the mitochondrial respiratory chain and from glycolysis. We have studied iron uptake in the rabbit reticulocyte when subjected to ethacrynic acid. The effect of this compound on ^{59}Fe uptake was observed in a high Na^+ environment (206 mosM NaCl) and a low Na^+ environment (6 mosM NaCl). The latter was the calculated amount contributed by rabbit serum added to an otherwise NaCl-free solution.

The procedure used was generally as follows. Cardiac blood (50–75 ml) was drawn from an adult rabbit which had previously been injected for several days with phenylhydrazine. The percentage of reticulocytes, determined by counting the number of cells stained with New Methylene Blue, was used to calculate the reticulocyte volume. In the experiment reported here the reticulocytes comprised 31 % of the cells. The final volume of cells is reported in terms of reticulocyte volume. The cells were washed 3 times (1000 × *g*, 10 min, 10°) in isotonic solution containing the following: 3 mosM $MgCl_2$, 200 mosM NaCl, 10 mosM KCl, 47 mosM choline chloride, and 50 mosM glycylglycine which buffered the solution at pH 7.4. When NaCl was omitted, choline chloride was substituted in equiosmolarity. Also, during incubation 10 mosM glucose replaced 10 mosM choline chloride.

Packed cells (0.8 ml) from the last centrifugation were suspended in 50 ml of the isotonic solution. Ethacrynic acid (Merck, Sharpe and Dohme) was present in the

experimental flask (0.1 mM). Absolute ethanol served as solvent and was present in the control flask (0.4 %). Finally 1 ml of commercially obtained rabbit serum containing ^{59}Fe was added to each flask. Previously, 2.6 μC of $^{59}FeCl$ (specific activity 20.2 $\mu C/\mu g$; New England Nuclear) were mixed with the serum and allowed to stand at least 1 h to allow for binding to transferrin. The amount of iron added was less than the calculated binding capacity.

A 2-ml aliquot of fluid for radioactive counting was taken from the flask 1 min after admixture. The flask containing the preparation was placed in a water bath at 37° and shaken constantly. Other aliquots for counting were taken at 15, 30, 60 and 90 min. Each aliquot was pipetted into an ice-cold solution in which serum was not present and nonradioactive iron quantitatively replaced ^{59}Fe. After centrifugation the supernatant was aspirated. After washing 2 times in this same solution the cells were hemolyzed with 20 mosM ice-cold Tris chloride (pH 8.0). The lysate was centrifuged (6600 × *g*, 20 min, 0°) in a Beckman L-2 Ultracentrifuge. The supernatant was decanted and an aliquot counted for radioactivity in a Nuclear-Chicago Automatic Scintillation Counter. The packed particulate layer (stroma) was dispersed in Triton X and counted for radioactivity. The data were converted to μg ^{59}Fe by reference to the calibrated source of ^{59}Fe.

The results of a representative study are as follows. As shown by the ^{59}Fe-uptake curves in Fig. 1, 0.1 mM ethacrynic acid after 15 min severely limited the quantity of iron bound to the stromal fraction. The effect was observed in both high and low Na^+ environments. Since the stromal fraction is taken to represent the membranous component of the cell it seems that the action of ethacrynic acid was to rapidly block surface receptor function. In contrast, in the control preparations, stromal uptake of ^{59}Fe was greater and increased during 90 min; but the rate was less after 30 min. Similarly, there was no essential difference in the controls when the experiment was conducted in the two types of Na^+ media.

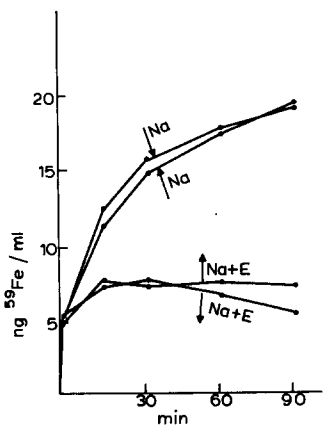

Fig. 1. Effect of 0.1 mM ethacrynic acid on ^{59}Fe bound by the reticulocyte stroma. Direction of the arrows denotes high and low Na^+, as explained in the text. E, ethacrynic acid. Ordinate is expressed in ng ^{59}Fe per ml compact reticulocytes.

The data in Table I demonstrate that 0.1 mM ethacrynic acid markedly restricted the amount of ^{59}Fe that partitioned in the supernatant after cell hemolysis.

TABLE I

ETHACRYNIC ACID (0.1 mM) AND SUPERNATANT ^{59}Fe

Data are expressed as ng ^{59}Fe per ml compact reticulocytes.

Time (min)	*Low Na^+*			*High Na^+*		
	(a) Control	*(b) Ethacrynic acid*	*Ratio (b/a)*	*(a) Control*	*(b) Ethacrynic acid*	*Ratio (b/a)*
1	3	3	1.00	3	3	1.00
15	44	15	0.34	54	19	0.35
30	88	28	0.32	106	32	0.30
60	162	41	0.25	186	45	0.24
90	221	48	0.22	245	53	0.22

The ratio of ethacrynic acid supernatant to control supernatant is shown in Table I for each interval of measurement. Comparison between the high and low Na^+ ratio's indicates that the inhibitory effect was independent of external Na^+. The progressive decline of this index indicates that ethacrynic acid not only maintained but slightly intensified its inhibitory action throughout incubation. The sharpest decline, however, occurred in the 1–15-min interval, indicating rapid onset of inhibition. The higher ^{59}Fe values in the high Na^+ supernatants indicate a favorable influence of Na^+ on iron movement from the stroma into the cell.

These experiments show that ethacrynic acid markedly decreased the stromal iron-binding capacity of rabbit reticulocytes. Also the compound caused less iron to be partitioned in the supernatant after cell hemolysis. It seems reasonable to assume that supernatant iron is iron that was initially attached to the stroma or membrane, and then moved into the cell. The ratios, presented in Table I, suggest that ethacrynic acid strongly impeded the rate of removal of ^{59}Fe from the stroma into the interior of the cell. The amount of ^{59}Fe in the supernatant apparently was not a direct function of the amount of ^{59}Fe attached to the stroma. Possibly this suggests that iron uptake by the reticulocyte consists of two steps which are distinct from each other but depend on a common source of energy that is blocked by ethacrynic acid. Unfortunately insufficient information is presently available about the precise biochemical action of ethacrynic acid to assess with confidence what its inhibitory action on iron uptake means in relation to an energy supplier.

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