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Induction of Intracellular ATP Synthesis by Extracellular Ferricyanide in Human Red Blood Cells

R. K. MISHRA and H. PASSOW

II. Physiologisches Institut, Universität des Saarlandes, 665 Homburg (Saar), Germany

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Summary. Human red blood cells rapidly convert extracellular ferricyanide into extracellular ferrocyanide. The reaction is enhanced by the addition of a substrate, adenosine. This increase of the rate of reaction is abolished by iodoacetate. The results indicate there is a flow of electrons across the membrane of metabolizing red blood cells. The reduction of extracellular ferricyanide is accompanied by the formation of intracellular ATP. The effect of an uncoupler and of inhibitors of oxidative phosphorylation on this reaction was studied under conditions where the natural rate of ATP synthesis was slightly reduced by 10^{-4} moles/liter iodoacetate. ATP formation was found to be inhibited by DNP, cyanide, and, to a lesser extent, by azide. Amytal is ineffective. Ferrocyanide enhances ATP breakdown. The action of DNP requires the presence of the cell membrane. It can probably not be related to a stimulation of the membrane ATPase of Laris and Letchworth, nor can it be explained on the basis of Mitchell's chemiosmotic hypothesis by effects on the passive permeability of the erythrocyte membrane to H^+ or alkali ions. In contrast to methylene blue and other oxidants, ferricyanide does not stimulate oxygen consumption in adult red blood cells.

In 1954 Manyai and Székely observed that the addition of ferricyanide to the medium of erythrocyte suspensions induced ATP synthesis inside the cells. Since the ferricyanide did not penetrate into the cells (Székely, Mányai and Straub, 1952), it was concluded that the oxidating agent effected ATP synthesis without direct contact with the glycolytic system. Mányai and Székely's observations were later confirmed and extended by Passow (1963) who showed, in addition, that ferrocyanide, in contrast to ferricyanide, induces ATP breakdown. More recently, Zamudio and Canessa (1966) were able to demonstrate the occurence of a ferricyanide reduced nicotinamide adenine dinucleotide (NADH) reductase in erythrocyte membrane preparations. Since such an enzyme complex could possibly participate in a transmembrane phosphorylation by ferricyanide, it seemed worth reviving the interest in the findings of the Hungarian workers. The present experiments were performed with the limited aim of providing additional evidence for the occurence of intracellular ATP synthesis and

the flow of electrons across the erythrocyte membrane in the presence of extracellular ferricyanide. We studied the use of agents which are known to uncouple or inhibit oxidative phosphorylation in mitochondria, and the results are thought to serve as a basis for future studies of the mechanism of electron transfer across cell membranes.

Materials and Methods

All experiments were performed with freshly drawn washed human erythrocytes. If not expressly stated otherwise, the cells were suspended in isotonic saline to give a cell concentration of 10% v/v. At zero time, the supernatant and the cells were mixed and samples were taken at suitable intervals for ATP analysis. In view of the low metabolic rate of red blood cells, the experiments usually lasted for about 2 hr. For ATP analysis, 3.0 ml of the suspension was withdrawn and precipitated by heating it in a boiling water bath for exactly 1.5 min. Subsequently, the suspension was cooled down to 0 °C and filtered. Control experiments with added ATP showed that heat precipitation did not result in any appreciable ATP breakdown. ATP analysis in the filtrate was performed by an enzymatic test involving phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase (Boehringer). Intracellular potassium was estimated by flame photometry after a measured volume of cell suspension was centrifuged at $1{,}600 \times g$ for 5 min and the sediment was diluted with distilled water. Erythrocyte membranes were prepared by a slight modification of the method of Weed, Reed, and Berg (1963). Phosphate was determined by the method of Behrenblum and Chain (1938) after butanol extraction. Changes of ferricyanide concentration in the supernatant could not be followed by simply measuring the light absorption at 405 mμ. Inevitable traces of hemolysis produced somewhat erratic readings. It was necessary, therefore, to employ a difference method. In a photometer cuvette, one drop of 0.16 M $NaNO_2$ was added to 2.5 ml of supernatant diluted 1:10 with distilled water, and the ensuing change of light extinction was recorded at 405 mμ. This yielded the concentration of ferricyanide in the sample. Subsequently, two drops of nearly saturated asorbic acid was added. The resulting change of light extinction was a measure for the sum of the concentrations of ferri- and ferrocyanide in the medium.

Results

When added to a suspension of cells metabolizing adenosine as a substrate, ferricyanide increases the rate of intracellular ATP formation. This effect is, however, not very marked. The action of the agent becomes much more apparent if the rate of ATP synthesis is reduced by the addition of small quantities of iodoacetate. In the presence of the inhibitor, the intracellular ATP content decreases in 2 hr to about 25 to 50% of its original value. Under these specific conditions, ferricyanide prevents net ATP breakdown almost completely (Fig. 1). The ferricyanide-induced ATP net synthesis may even lead to ATP levels exceeding those at the start of the experiment. Ferrocyanide accelerates ATP breakdown.

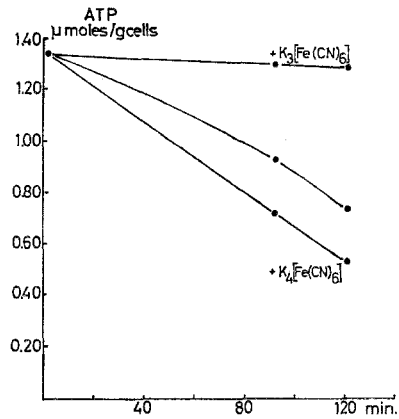

Fig. 1. Effects of ferricyanide and ferrocyanide on ATP content of human red blood cells. Composition of medium: iodoacetate, 0.125 mmoles/liter; and adenosine, 5mmoles/liter, in isotonic saline (unmarked curve). The other two curves represent experiments where the medium contained (in addition to these constituents): ferricyanide [$K_3Fe(CN)_6$], 1 mmole/liter, or ferrocyanide [$K_4Fe(CN)_6$], 1 mmole/liter. Cell concentration: 10% v/v. 37 °C. Ordinate: ATP content of cells in µmoles/g initial weight. Abscissa: time in minutes

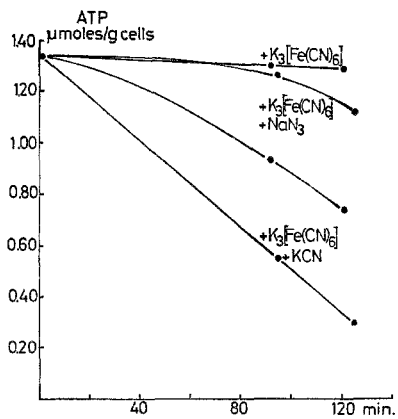

Fig. 2. Effects of azide and cyanide on ferricyanide-stimulated ATP synthesis in iodoacetate-poisoned red blood cells. The media contained: iodoacetate, 0.125 mmoles/liter; adenosine, 5 mmoles/liter (curve not marked); ferricyanide [+$K_3Fe(CN)_6$], 1 mmole/liter, and either NaN_3[+$K_3Fe(CN)_6$+NaN_3], 1 mmole/liter, or KCN[+$K_3Fe(CN)_6$+KCN], 1 mmole/liter. Cell concentration: 10% v/v. 37 °C. Ordinate: ATP content of cells in µmoles/g initial weight. Abscissa: time in minutes

The actions of a number of inhibitors of electron transfer (cyanide, azide, and amytal), as well as of the uncoupler dinitrophenol (DNP), on the effects of ferricyanide and ferrocyanide were tested in iodoacetate-poisoned cells consuming adenosine as a substrate (Fig. 2). Amytal had no

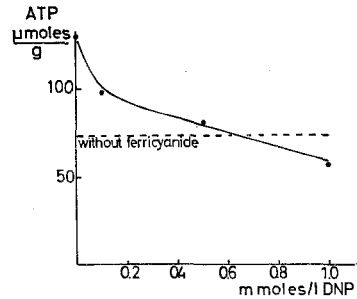

Fig. 3. Effects of DNP on ferricyanide-stimulated ATP generation in iodoacetate-poisoned red blood cells. The medium contained: iodoacetate, 0.125 mmoles/liter; adenosine, 5 mmoles/liter; $K_3Fe(CN)_6$, 1 mmole/liter, and varying concentrations of DNP. Ordinate: ATP content of cells (µmoles/g initial weight) after 90 min of incubation at 37 °C. Abscissa: DNP concentration in the medium. Dashed line: ATP content of cells incubated 90 min in a medium of the composition indicated above except that neither ferricyanide nor DNP was present

effect on ATP synthesis in the presence of ferricyanide. Azide exerted a slight inhibitory effect, cyanide a strong. If applied in sufficiently high concentrations, cyanide could even increase the rate of ATP splitting beyond the control value obtained with iodoacetate in the absence of ferricyanide. DNP is also capable of preventing ferricyanide-induced intracellular ATP synthesis. The efficiency of DNP increases with increasing concentration. As is the case with cyanide, at high concentrations (0.6 mM or more) DNP induces ATP breakdown at a rate which exceeds the rate observed in the absence of ferricyanide (Fig. 3).

Ferricyanide does not penetrate into the cells. This is obvious because the brownish discoloration due to methemoglobin formation which occurs in hemolysates immediately upon addition of ferricyanide does not take place. This qualitative observation is substantiated by quantitative determinations of the sum of ferri- and ferrocyanide in the suspension medium. Within the limits of experimental accuracy, no penetration of either $[Fe(CN)_6]^{3-}$ or $[Fe(CN)_6]^{4-}$ could be detected. In spite of the impermeability of the membrane, ferricyanide is rapidly reduced to ferrocyanide. In the experiment represented in Fig. 4, in the absence of a substrate, about 45 % of the extracellular ferricyanide (or about 4 µmoles/g red cells) is converted into extracellular ferrocyanide. If a substrate is added, the rate of ferricyanide reduction is approximately doubled. The effect of the substrate is abolished by iodoacetate. These experiments clearly demonstrate that extracellular ferricyanide acts as an acceptor of electrons which originate from metabolic reactions occuring in the cell interior.

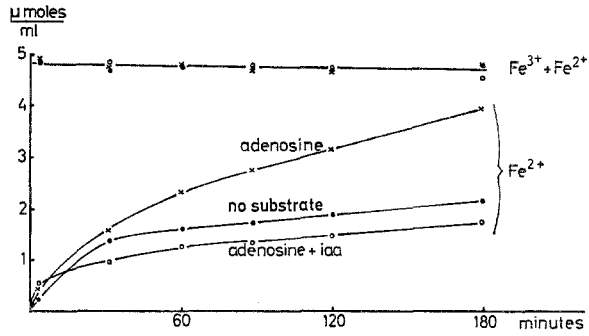

Fig. 4. Time course of ferrocyanide formation from ferricyanide in the supernatant of a red blood cell suspension (curves marked Fe^{2+}). The media contained ferricyanide, 2.0 mmoles/liter (filled circles) and the following additions: adenosine, 5 mmoles/liter, (crosses) or iodoacetate, 0.1 mmole/liter and adenosine, 5 mmoles/liter (hollow circles). The upper curve ($Fe^{3+}+Fe^{2+}$) shows for the three experiments the time course of changes of the sum ferricyanide plus ferrocyanide in the supernatant after correction for small changes of hematocrit. Cell concentration: 44% v/v. 37 °C. Ordinate: concentration in the supernatant in mmoles/liter. Abscissa: time in minutes

Table 1. *Amount of ferricyanide reduced by iodoacetate-poisoned red blood cells which metabolize adenosine* [a]

Ferricyanide reduced		Time
Without α-DNP µmoles/g	With α-DNP µmoles/g	min
0.63	0.52	3
1.01	1.06	16
1.46	1.40	32
1.72	1.76	58
1.98	2.08	90
2.10	2.17	121
2.28	2.35	152
2.35	2.42	182

[a] Composition of medium: ferricyanide, 1 mmole/liter; iodoacetate, 0.1 mmole/liter; adenosine, 5 mmoles/liter; DNP, 0.5 mmoles/liter; and NaCl, 166 mmoles/liter.

In the presence of DNP, the flow of electrons across the membrane continues at virtually the same rate as in its absence (Table 1). At first glance, this finding would suggest a fundamental difference between the actions of DNP on electron transfer across the red cell membrane and on oxidative phosphorylation in mitochondria. However, closer inspection of our data makes such a conclusion appear premature. The synthesis of 1 µmole ATP in the cells is associated with the reduction of about 4 to

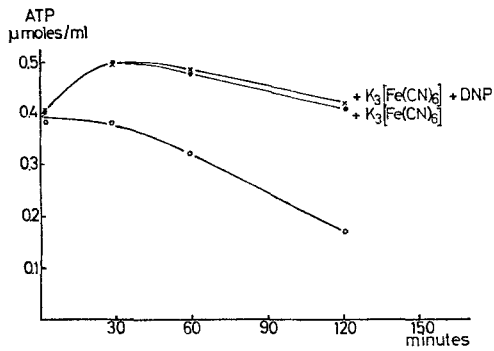

Fig. 5. Effects of ferricyanide and ferricyanide plus DNP on ATP synthesis in a stroma-free hemolysate. The hemolysate was obtained by osmotic hemolysis of one part of cells in four parts of distilled water and by subsequent freezing and thawing of the hemolysate for three times. The membranes were removed by centrifugation at $33{,}000 \times g$ for 15 min. The following substances were added to the hemolysate: KCl (100), NaCl (66), $MgCl_2$ (2.0), Na_2HPO_4 (0.3), adenosine (5.0), ADP (1.0), NAD (1.0), and iodoacetate (0.1). The figures in brackets represent the final concentrations (mM) in the hemolysate. The curves marked $+K_3Fe(CN)_6$ and $+K_3Fe(CN)_6+DNP$ were obtained in experiments where the medium contained (in addition to the listed constituents) ferricyanide, 5.0 mmoles/liter, or ferricyanide, 5.0 mmoles/liter, plus DNP, 0.2 mmoles/liter respectively. Similar results are obtained if pyruvate is used in place of ferricyanide. Ordinate: ATP content of a measured volume, in µmoles. Abscissa: time in minutes

8 µmoles of ferricyanide. Perhaps only a very small fraction of the total electron flow is involved in the "oxidative phosphorylation" of ADP. "Uncoupling" of this fraction may lead only to an insignificant increase of the measured total ferricyanide reduction and thus escape detection.

The inhibitory action of DNP on ATP synthesis requires the presence of the membrane. When added to an adenosine-containing hemolysate from which the membranes were first removed by centrifugation, ferricyanide is capable of augmenting ATP synthesis as in intact cells. However, this effect cannot be abolished by the addition of DNP (Fig. 5).

Laris and Letchworth (1967) have observed a stimulation of the release of inorganic phosphate by DNP in intact human red cells. They relate this finding to the activation by DNP of a membrane ATPase. Clearly, a DNP-stimulated ATPase could be responsible for the suppression of the ferricyanide-induced ATP net synthesis by DNP. Like ferricyanide, the easily penetrating pyruvate can raise the intracellular ATP levels of iodoacetate-poisoned red cells. However, in contrast to the action of ferricyanide, the pyruvate-induced ATP synthesis cannot be inhibited by DNP (Fig. 6). This finding suggests that the DNP-sensitive ATPase described by Laris and Letchworth is unlikely to account for our observations. It

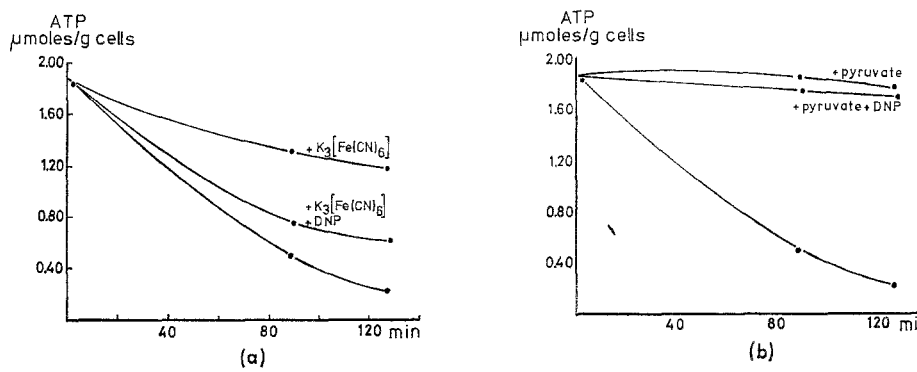

Fig. 6. Effect of DNP on ATP synthesis in the presence of ferricyanide (a) or pyruvate (b). Composition of media: iodoacetate, 0.125 mmoles/liter, and adenosine, 5 mmoles/liter, in isotonic saline (unmarked curves. (a) In addition to iodoacetate and adenosine, the media contained: ferricyanide, 1 mmole/liter [curve $K_3Fe(CN)_6$]; or ferricyanide, 1 mmole/liter, plus DNP, 0.3 mmoles/liter [curve $K_3Fe(CN)_6$ + DNP]. (b) In addition to iodoacetate and adenosine, the media contained: pyruvate, 1 mmole/liter (curve pyruvate); or pyruvate, 1 mmole/liter, plus DNP, 0.2 mmoles/liter (curve pyruvate + DNP). Cell concentration: 10% v/v. 37 °C. Ordinate: ATP content of cells in μmoles/g initial weight. Abscissa: time in minutes

also confirms our contention that it is extracellular rather than an undetected trace of intracellular ferricyanide which induces ATP synthesis.

According to Mitchell (Mitchell & Moyle, 1967), the maintenance of a stationary nonequilibrium distribution of H^+ ions across the membrane represents one of the driving forces of oxidative phosphorylation in mitochondria. DNP is supposed to increase the passive permeability to H^+ ions and hence to diminish the driving force for phosphorylation. A similar explanation cannot be applied to our observations with a cell membrane; the red cell is highly permeable to anions, including OH^-. Since the dissociation equilibrium between OH^- and H^+ is quickly established, OH^- as well as H^+ ion distribution between cells and medium always represents a Donnan equilibrium which cannot serve as a source of energy for phosphorylation. Hence, a further DNP-induced increase of the passive permeability to H^+ ions should have no effect on phosphorylation.

Although the downhill movement of H^+ ions is unlikely to be involved in ATP synthesis of the red cell, it seems conceivable that a flow of potassium and sodium through the pump down their electrochemical gradients could be associated with the generation of ATP in the presence of iodoacetate and ferricyanide. Such a hypothesis does not seem to be implausible since it is well known (Gruner and Passow, 1963; Passow, 1964) that the combined application of iodoacetate and ferricyanide evokes a considerable

increase of passive potassium fluxes in human red cells. However, under the conditions of the present experiments, the iodoacetate concentration was only about 10% of that employed by Gruner and Passow, and, thus, little potassium loss occured under conditions where a rapid ATP synthesis took place. Moreover, if the cells are suspended in media of nearly the same K^+ and Na^+ concentration as inside the cells, the driving forces for passive net potassium and sodium movements are negligible, and yet ferricyanide induces ATP synthesis and DNP blocks this effect just as in NaCl solution where large concentration gradients exist. The experimental results are indistinguishable from those depicted in Fig. 1.

Discussion

The augmentation by ferricyanide and pyruvate of ATP synthesis in membrane-free hemolysates can probably be easily explained. In hemolysates, the ribose moiety of adenosine is converted via the pentose phosphate pathway into glyceraldehyde phosphate (*see* Marks, 1964). In the presence of partially inhibitory concentrations of iodoacetate, for the conversion of this substrate into lactate, the glyceraldehyde phosphate dehydrogenase should be rate limiting. Addition of ferricyanide or pyruvate should shift the equilibrium between NAD and NADH and should favor the formation of NAD. This would lead to an activation of the partially blocked glyceraldehyde phosphate dehydrogenase, and hence to a restoration of the activity of this enzyme. This reaction cannot, of course, be inhibited by DNP. The penetration into the cells of traces of ferricyanide which were not detected by our analytical procedure could, conceivably, act in this way. Such an explanation of the ferricyanide action is, however, unlikely because: (i) methemoglobin formation cannot be demonstrated, and (ii) the inhibition by DNP of phosphorylation requires the presence of the membrane.

If intracellular NADH would act as an electron donor and extracellular ferricyanide as an acceptor, an electron transfer across the membrane would lead to a partial restoration of the glyceraldehyde phosphate dehydrogenase activity by the mechanism described above. However, the membrane ferricyanide NADH-dehydrogenase described by Zamudio and Canessa (1966) cannot be inhibited by either DNP or cyanide *(unpublished observation)*. This would suggest that an electron transfer only by means of this flavine enzyme cannot account for our observations.

A variety of substances including methylene blue which can potentially cause the oxidation of reduced nicotinamide adenine dinucleotide phos-

phate, (NADPH) are known to stimulate the oxidation of glucose-6-phosphate in the red cell (*see* Marks, 1964). The products of oxidation are channeled into the pentose phosphate pathway and may aid the formation of ATP. Methylene blue induces a considerable increase of O_2 uptake in cells which use adenosine instead of glucose as a substrate (Table 2).

Table 2. *Oxygen consumption of human red blood cells without additions, with methylene blue (0.5 mmoles/liter), or with ferricyanide (2.0 mmoles/liter). The cells (20% v/v) are in saline containing 5 mmoles/liter of either glucose or adenosine as substrate. No iodoacetate is present. The Warburg technique was used*

Substrate	O_2 Consumption		
	Without additions µliters	With methylene blue µliters	With $K_3[Fe(CN)_6]$ µliters
Glucose	45.1	206.5	50.4
Adenosine	30.9	206.0	25.8

Obviously, the products of ribose phosphate metabolism include hexose phosphates which are known to be oxidized in the presence of the redox dye. The action of ferricyanide on ATP synthesis cannot, however, be related to a stimulation of the conversion of NADPH into NADP and the concomitant oxidation of glucose. In contrast to methylene blue, ferricyanide does not affect O_2 consumption of red blood cells, regardless of whether the cells use glucose or adenosine as a substrate.

So far we have only considered the possibility that ferricyanide enhances the partially blocked glycolysis by oxidation of NADH or NADPH, and thus maintains ATP synthesis at a nearly normal rate. Alternatively, it may be worth asking if the flow of electrons across the membrane is directly coupled to an incorporation of inorganic phosphate into ADP. On the basis of the present material, this question cannot be answered definitely. The effect of DNP on electron flow is difficult to interpret. Moreover, in experiments with erythrocyte ghosts or broken cell membranes which are virtually free of hemoglobin and other intracellular compounds, we have tried to demonstrate ATP synthesis from ADP and inorganic phosphate. In the presence of ferricyanide as an electron acceptor and of NADH as a donor, no phosphorylation could be observed, although under these experimental conditions NADH was readily oxidized by the ferricyanide-NADH dehydrogenase described by Zamudio and Canessa (1966). Perhaps ATP synthesis can only be accomplished if the presence of ferricyanide is confined to the outside of the membrane so that the agent can induce a vectorial flow of electrons, or if some cytoplasmic factor is present.

In conclusion, the substrate-dependent iodoacetate-inhibitable ferricyanide reduction by red blood cells indicates that the erythrocyte membrane possesses the means of transferring electrons from its inside to the outside. Without inhibiting the flow of electrons – but apparently also without enhancing it – DNP prevents the induction of intracellular ATP synthesis by extracellular ferricyanide. The mechanism of the electron transfer as well as its relation to the concomitant ATP synthesis remains to be elucidated. For the reason discussed above, it is unlikely that ATP synthesis is increased solely by the conversion of intracellular NADH into NAD and the ensuing stimulation of glycolysis. The possibility exists, therefore, that ATP is synthesized in the membrane by a reaction which is linked to the observed electron transfer. However, the action of DNP on such a coupling cannot be explained on the basis of Mitchell's (Mitchell & Moyle, 1967) chemiosmotic hypothesis or the related assumptions that passive movements of alkali ions generate ATP when they move down their electrochemical gradients and that DNP abolishes these gradients. DNP does not induce an ATPase activity in cells whose ATP synthesis is stimulated by easily penetrating pyruvate. However, in the presence of ferricyanide, DNP can increase the rate of ATP hydrolysis in the cells even above the value observed in the absence of ferricyanide. Since the participation of cytochromes in the electron transfer across the membrane of mature red blood cells appears unlikely, the mode of action of cyanide and azide remains as obscure as that of DNP.

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