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THE ENZYMATIC REDUCTION OF FERRIHEMOGLOBIN

I. THE REDUCTION OF FERRIHEMOGLOBIN IN RED BLOOD CELLS AND HEMOLYSATES

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SUMMARY

1. Reduction of ferrihemoglobin by NADH in hemolysates is shown to be strongly dependent on the oxidizing agent used to produce ferrihemoglobin from hemoglobin.

2. Oxidation of hemolysates by ferricyanide resulted in preparations in which ferrihemoglobin was very rapidly reduced by NADH.

3. This activation is interpreted as due to the binding of ferrocyanide to ferrihemoglobin. Evidence is presented that ferrocyanide does not act as an electron carrier.

4. It is suggested that ferrocyanide binding induces a change in the ferrihemoglobin structure, which permits "NADH–ferrihemoglobin reductase" to approach the site of reduction more freely.

INTRODUCTION

It is generally accepted that the red blood cells possess enzymatic mechanisms for the maintenance of hemoglobin in its reduced, active state[8,9,13,15,16,19,22]. It seems also well established that this ferrihemoglobin reduction is dependent on the regeneration of reduced pyridine nucleotides[8,9,15]. Red blood cells incubated with glucose or other substances that can be metabolized in erythrocytes by reactions leading to the production of reduced pyridine nucleotides, will promote the reduction of ferrihemoglobin[14]. Hemolysates, on the other hand, were found to be considerably less active in this reduction even in the presence of high concentrations of reduced pyridine nucleotides[3,9,15].

This paper describes several experimental conditions for the assay of ferri-

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hemoglobin reduction in hemolysates. Conditions are described under which extremely high rates of ferrihemoglobin reduction could be measured.

MATERIALS AND METHODS

Materials

NADH (Grade III), NADPH (Type II), phenazine methosulphate (Grade III), were purchased from the Sigma Chemical Company. Methylene blue was a preparation of Eastman, Organic Chemicals Department. Dowex AG 1-X10 (analytical grade, chloride form; 200–400 mesh) was a product of the BioRad Corporation for Biochemical Research. Sephadex was purchased from Pharmacia, Uppsala, Sweden.

All other chemicals used were of the purest grade commercially available.

Preparations

Prep. 1. Hemolysate containing ferrohemoglobin

The method of HENNESSEY *et al.*[11] which utilizes toluol and water for hemolysis was used.

Prep. 2: "Ferricyanide hemolysate"

Hemolysates from fresh human erythrocytes *(Prep. 1)* were treated with 1.2 mole of $K_3Fe(CN)_6$ per mole hemoglobin iron, using a 0.05 M solution of $K_3Fe(CN)_6$ in distilled water. After allowing 10 min for oxidation of the pigment, 5 ml of the mixture were applied to a column of Sephadex G-25 (coarse, 3 cm × 30 cm). The gel was pretreated by washing the granules several times with 0.001 M NaCl. Gel filtration was performed at room temperature with the same 0.001 M NaCl. Two clearly defined and well separated bands were evident on the column: a lower, brown band consisting primarily of ferrihemoglobin and an upper yellow band of ferricyanide. The ferrihemoglobin band, eluted separately, was used in the experiments and will be referred to as "ferricyanide hemolysate".

Prep. 3: "Dowex" hemolysate ("ferricyanide hemolysate" treated by Dowex 1 anion-exchange resin)

10 ml "ferricyanide hemolysate" followed by 6 ml of distilled water were passed through a column (1 cm × 5 cm) Dowex AG-1 anion-exchange resin, chloride form. A pale yellow band containing ferri- and ferrocyanide remained strongly attached to the top of the column. The eluate, ferrihemoglobinemic hemolysate, which was analysed and shown to be free of ferri- and ferrocyanide will be referred to as "Dowex hemolysate".

Assay 1: Ferrihemoglobin reduction in hemolysates containing 0.1 μmole ferrihemoglobin per ml. The reaction mixture contained in μmoles/ml: ferrihemoglobin, added as hemolysate, 0.1; nicotinamide, 1.0; EDTA (disodium salt), 0.5; Tris–HCl buffer (pH 7.35), 25.0; NADH (or NADPH), 0.1. The NADH (or NADPH) was added at zero time and the reaction was followed at 575 mμ by use of a $\Delta\varepsilon$ $42.0 \cdot 10^3$ (ref. 10) for the difference between ferri- and ferrohemoglobin. A blank solution, containing all additions except NADH (or NADPH) was used. Measurements were carried out at 5-min intervals. The actual pH of the reaction mixture fluctuated from 6.8 to 7.0.

Using a Beckman DU spectrophotometer, changes in ferrihemoglobin (converted to ferrohemoglobin) down to 0.5 mμmole/ml could be accurately determined.

Assay 2: Ferrihemoglobin reduction in hemolysates containing 0.025 μmole ferrihemoglobin per ml. The reaction mixture contained in μmoles/ml: hemolysate containing ferrihemoglobin, 0.025; EDTA (disodium salt), 0.5; nicotinamide, 1.0; citrate buffer (pH 5.0), 3.0; NADH, 0.1. NADH was added at zero time and the change in absorbance was measured at 575 mμ at 30-sec intervals against a blank, containing all additions, except NADH. The pH of the reaction mixture was around 5.2. Several variations of Assay 2 involving the preparation of ferrihemoglobin and the incorporation of activators into the reaction mixture were employed. These modifications are indicated under the corresponding tables and figures.

In both assays, the initial rates of reduction were used to calculate results. They were expressed as μmoles ferrihemoglobin reduced per min per mg total protein determined as hemoglobin.

Other analytical methods

Total hemoglobin was determined by the method of CANNAN[4] using a commercial cyanferrihemoglobin standard, prepared by Ortho Pharmaceutical Corp., Raritan, N.J., and ferrihemoglobin by the method of EVELYN AND MALLOY[6]. A molar extinction coefficient of $6.22 \cdot 10^3$ at 340 mμ was used to calculate pyridine nucleotide concentrations. Nitrites were determined by the method of STIEGLITZ AND PALMER[20]. For the determination of ferrocyanide in presence of ferricyanide the method of AVRON AND SHAVIT[2] was used. Ferricyanide in the presence of ferrocyanide was determined by the following procedure: equal amounts of hemolysate and 14% trichloroacetic acid were mixed, heated for 30 min in a boiling-water bath and the precipitate was removed by centrifugation. The absorbance of the clear supernatant was measured at 420 mμ before and after the addition of a few crystals of ascorbic acid. A molar extinction coefficient of $1.03 \cdot 10^3$ (ref. 17) for ferrocyanide absorption was used. Ultracentrifugations were performed in a Beckman Spinco analytical ultracentrifuge, Model E. All spectrophotometric measurements were performed with a Beckman DU spectrophotometer except the continuous absorption spectra which were recorded on a Cary recording spectrophotometer, Model 14. Quartz cells with a 1-cm light path were generally used.

RESULTS

The reduction of ferrihemoglobin in whole erythrocytes and hemolysates

The method of ROSSI-FANELLI[18] was used to measure reduction in whole nitrite-treated erythrocytes. Glucose and lactate were approximately equally effective in promoting ferrihemoglobin reduction, the rates being $2.6 \cdot 10^{-5}$ for glucose and $3.1 \cdot 10^{-5}$ for lactate. The considerable accelerating effect of methylene blue, especially with glucose as substrate, was also observed (rate of reduction, $20.0 \cdot 10^{-5}$). These results are in agreement with the findings of earlier investigators[8,15,18,22,23].

Reduction by NADH was found to be considerably slower in hemolysates than in whole cells. The method of SCOTT[19], which simulates conditions inside whole cells, was used. Rates of reduction of $0.7 \cdot 10^{-5}$ for NADH and $0.3 \cdot 10^{-5}$ for NADPH were measured.

A simpler direct spectrophotometric assay for measuring rates of ferrihemoglobin reduction was developed. Much lower concentrations of ferrihemoglobin had

TABLE I

RATE OF FERRIHEMOGLOBIN REDUCTION BY DIFFERENT REDUCING SYSTEMS IN HEMOLYSATES PREPARED FROM NITRITE-TREATED ERYTHROCYTES

Assay 1 was used. The reaction mixture contained: ferrihemoglobin (added as hemolysate), $1 \cdot 10^{-4}$ M; nicotinamide, $1 \cdot 10^{-3}$ M; Tris-HCl buffer (pH 7.55), $2.5 \cdot 10^{-2}$ M; EDTA (disodium salt), $5 \cdot 10^{-4}$ M; NADH or NADPH, $1 \cdot 10^{-4}$ M; methylene blue, $1 \cdot 10^{-5}$ M; phenazine methosulfate, $1 \cdot 10^{-5}$ M. Hemolysates were prepared as described by ROSSI-FANELLI, ANTONINI AND MONDOVI[18].

Expt. No.	*Reducing system*	*Ferrihemoglobin reduction (μmoles/min per mg protein × 10⁵)*
1	None	0.0
2	NADH	0.2
3	NADPH	0.1
4	NADH + methylene blue	6.5
5	NADPH + methylene blue	14.7
6	NADH + phenazine methosulphate	326
7	NADPH + phenazine methosulphate	690
8	NADH*	0.0
9	NADH + methylene blue*	0.0
10	NADH + phenazine methosulphate*	331

* Hemolysate heated at 56° for 1 h before its incorporation into the reaction mixture.

to be used in this procedure (Assay 1). Table I presents measurements at a ferrihemoglobin concentration of $1 \cdot 10^{-4}$ M. This concentration represents a close approximation to the ferrihemoglobin concentration in normal human erythrocytes (where only approx. 2% of the hemoglobin is in its oxidized form).

The activity under these conditions was even lower than in the concentrated hemolysates (used in SCOTT's method). As shown by COX AND WENDEL[5] and KIESE[15], rates of ferrihemoglobin reduction decrease rapidly in this concentration region with decreasing concentrations of the pigment. Addition of methylene blue increased the rate considerably, this being more with NADPH than with NADH (Table I, 4, 5). In an examination of other electron carriers, we found that phenazine methosulphate accelerated the rate of reduction even more (Table I, 6, 7). Heating the hemolysate for 1 h at 56° completely stopped ferrihemoglobin reduction by NADH or by NADH *plus* methylene blue (Table I, 8, 9), suggesting the enzymatic nature of both reactions. However, in the presence of phenazine methosulfate (Table I, 10), activity was not destroyed by heat treatment. Thus, phenazine methosulfate probably acts non-enzymatically.

The reduction of ferrihemoglobin in hemolysates in which ferrihemoglobin was formed with ferricyanide

In view of the fact that the rate of reduction of ferrihemoglobin by NADH and NADPH in hemolysates was found to be considerably slower than in whole cells, it was thought possible that the nitrite treatment might harm the reduction system, the effect demonstrating itself mainly after hemolysis. Therefore, another oxidant, ferricyanide, was tried.

Hemolysates (Prep. 1) were treated with an excess of potassium ferricyanide

TABLE II

INFLUENCE OF DIFFERENT TREATMENTS ON THE RATE OF REDUCTION OF FERRIHEMOGLOBIN BY NADH IN HEMOLYSATES PREPARED BY FERRICYANIDE OR NITRITE

Rates of reduction were measured by Assay 2. In Expts. 1–7, $K_3Fe(CN)_6$ was added in large excess.

Expt. No.	*Treatment of hemolysate*	*Ferrihemoglobin reduction (μmoles/min per mg protein × 10^5)*
1	$K_3Fe(CN)_6$	0.0
2	$K_3Fe(CN)_6$ + dialysis against distilled water for 6 days	220
3	$K_3Fe(CN)_6$ + dialysis against 0.9% NaCl for 6 days	14.4
4	$K_3Fe(CN)_6$ + Sephadex column	238
5	$K_3Fe(CN)_6$ + Sephadex column + heating, 56°, 1 h	0.0
6	$K_3Fe(CN)_6$ + Sephadex column + Dowex column	0.2
7	$K_3Fe(CN)_6$ + Sephadex column + Dowex column + $K_4Fe(CN)_6$ in a 4:1 molar ratio to ferrihemoglobin	210
8	$K_3Fe(CN)_6$ in a 4:1 molar ratio to hemoglobin	302
9	Nitrite	0.0
10	Nitrite + $K_4Fe(CN)_6$ in a 4:1 molar ratio to ferrihemoglobin	216

and dialyzed against distilled water until no ferricyanide could be detected in the dialysing fluid. Upon addition of NADH very high rates of ferrihemoglobin reduction could be measured (Table II, 2). 6–7 days of dialysis were necessary to achieve maximal activation. The rates were so high that in order to measure them spectrophotometrically with reasonable accuracy a method (Assay 2) using a much lower concentration of ferrihemoglobin (0.025 μmole/ml) had to be developed. When the ferricyanide was not dialysed out, no reduction could be observed, probably because the excess ferricyanide served itself as an electron acceptor (Table II, 1; see also Fig. 1B). Dialysis of the active preparation against 0.9% NaCl caused a fall in rates of reduction, which was accompanied by the release of potassium ferrocyanide into the dialysing medium (Table II, 3). To eliminate inactivation during the prolonged dialysis, the separation of ferricyanide from ferrihemoglobin was attempted on a Sephadex column (Prep. 2). The "ferricyanide–ferrihemoglobin" preparation so produced reacted extremely rapidly with NADH: 15% of the pigment was reduced in the first minute after addition of the reduced pyridine nucleotide (Table II, 4). The reduction appeared enzymatic in nature, as was indicated by the fact that heating at 56° for 1 h resulted in the complete loss of activity (Table II, 5).

GIBSON[7] reported that ferrocyanide accelerated the rate of the non-enzymatic reduction of ferrihemoglobin by ascorbate. Therefore, we tried to find out whether the high rates found with the "ferricyanide–ferrihemoglobin" preparations were also due to a catalytic effect of ferrocyanide produced from ferricyanide in the oxidation reaction with hemoglobin. This assumed that some of the added ferricyanide remained bound as ferrocyanide to the ferrihemoglobin in spite of the clear separation on the Sephadex column. To test this assumption we passed the "ferricyanide–ferrihemoglobin" preparation through a Dowex 1 column in order to remove ferro- or ferricyanide (see Prep. 3). Indeed, the rate of reduction by NADH dropped essentially to zero (Table II, 6). Complete activity could be restored by adding ferrocyanide (Table II, 7) to the reaction mixture.

A most active "ferricyanide–ferrihemoglobin" preparation could be obtained without Sephadex treatment or dialysis. When ferricyanide was added directly to a hemolysate (Prep. 1) in a 4:1 molar ratio to hemoglobin, complete conversion to ferrihemoglobin and binding of the resulting ferrocyanide was achieved simultaneously. The specific activity measured with this preparation was $302 \cdot 10^{-5}$ (Table II, 8). The rate of ferrihemoglobin reduction in hemolysates prepared from nitrite-treated erythrocytes[18], determined under the same experimental conditions, is presented for comparison (Table II, 9). As shown, at such a low concentration of ferrihemoglobin (0.025 μmole/ml) no measurable reduction by NADH could be demonstrated with this preparation. However, addition of ferrocyanide to the hemolysate restored activity (Table II, 10).

The evidence presented so far is interpreted as follows: On oxidation of hemoglobin by ferricyanide, ferrocyanide was formed and bound to the ferrihemoglobin molecule. This resulted in a preparation which was highly active in reducing ferrihemoglobin by NADH. Oxidation of hemoglobin by nitrite does not result in such an activation.

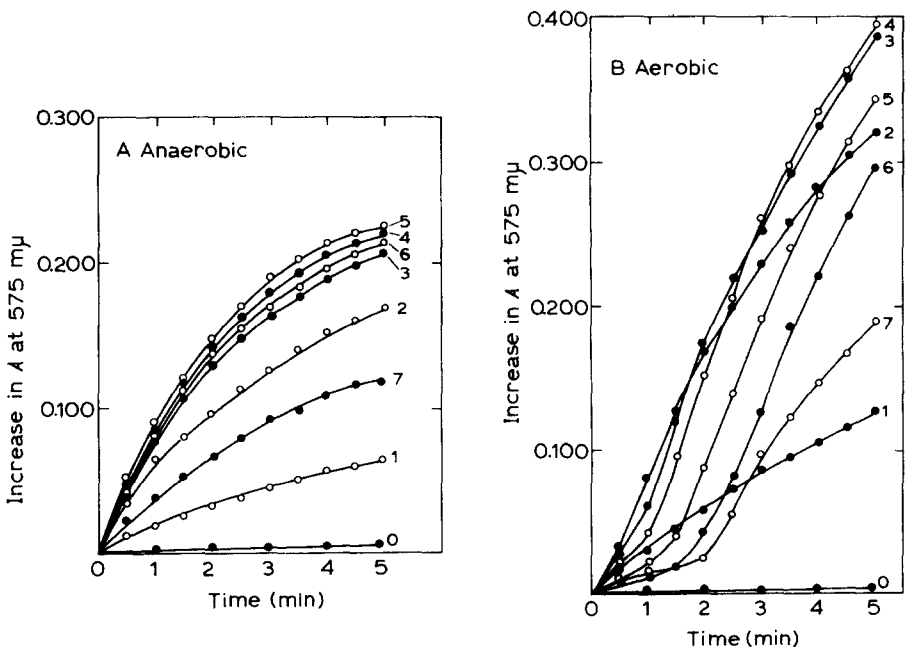

Fig. 1. Rate of ferrihemoglobin reduction in "Dowex hemolysates" as a function of added ferrocyanide. Rates were measured by Assay 2. Numbers represent molar ratios of ferrocyanide to ferrihemoglobin. Experiments were done anaerobically (A) and aerobically (B). Thunberg tubes, fused to glass cuvettes were used for the anaerobic work. NADH was added from the side arm.

The quantitative action of potassium ferrocyanide as an activating substance

Figs. 1A and B demonstrate the activation of the ferrihemoglobin reduction system by adding different amounts of ferrocyanide to "Dowex hemolysates". Under anaerobic conditions rates were proportional to the amount of ferrocyanide added up to a molar ratio of about 4:1 for ferrocyanide: ferrihemoglobin. Addition

of more ferrocyanide did not increase rates, but considerable excess had an inhibitory effect. Under aerobic conditions (Fig. 1B) the effect was similar, except for the appearance of "lags" in the curves representing reduction as function of time. The "lags" lengthened considerably when ferrocyanide was added in excess of a molar ratio of 4:1, and were proportional to the excess added. They could be explained by assuming that up to a ratio of 4:1, ferrocyanide is attached in some way to ferrihemoglobin and, added in excess, it shifts the equilibrium:

$$\text{Ferrocyanide} + \text{ferrihemoglobin} + O_2 \rightleftharpoons \text{Ferricyanide} + \text{oxyhemoglobin}$$

to the right. The ferricyanide thus produced exerts a delaying effect on the reduction reaction by converting newly formed oxyhemoglobin to ferrihemoglobin. This effect demonstrated itself as a "lag", whose slope diminished with time as a consequence of the progressive decrease in the concentrations of ferricyanide and ferrihemoglobin and the increase in the concentration of ferrocyanide and oxyhemoglobin in the reaction mixture. Alternatively, ferricyanide could serve as a direct electron acceptor for the ferrihemoglobin-reducing enzyme.

Comparing the curves in Figs. 1A and B, it can be seen that for equal molar ratios, ΔA per min values were considerably different for aerobic and anaerobic conditions. However, it must be considered that under anaerobic conditions the change measured was $A_{\text{hemoglobin}} - A_{\text{ferrihemoglobin}}$, while under aerobic conditions it was $A_{\text{oxyhemoglobin}} - A_{\text{ferrihemoglobin}}$. HORECKER[12] found that the ratio $(A_{\text{oxyhemoglobin}} - A_{\text{ferrihemoglobin}})/(A_{\text{hemoglobin}} - A_{\text{ferrihemoglobin}})$ is 2.1. When corrected for this factor, the rates of reduction were identical under aerobic and anaerobic conditions. This was confirmed experimentally. The Thunberg tubes were aerated at the end of experiments done anaerobically and the A values measured again. The data obtained were in full agreement with the above factor of 2.1.

The binding of ferrocyanide to ferrihemoglobin

As pointed out earlier, the experiments summarized in Table II and Fig. 1 pointed towards a binding between ferrocyanide and ferrihemoglobin, producing a complex responsible for the high rates of reduction. Additional evidence supporting the existence of such a complex was sought. Table III summarizes the results of an experiment in which different concentrations of ferricyanide were added to a hemolysate and where the resulting solution was passed through a Sephadex column.

It can be seen that for every mole of ferricyanide, one-fourth of a mole of ferrihemoglobin was formed almost stoichiometrically (Table III, 1–3). Regardless of whether a great, small or no excess of ferricyanide was used for the complete oxidation of the pigment in the preparation (Table III, 3–5), the rate of ferrihemoglobin reduction after the Sephadex treatment was practically the same, suggesting that definite amounts of the catalytic agent remained bound to the ferrihemoglobin band, the excess of ferricyanide being separated on the Sephadex column.

Further support confirming the production of a ferrocyanide–ferrihemoglobin complex was obtained in equilibrium dialysis experiments. Hemolysate was dialysed against a dilute solution of ferricyanide. After 72 h, the concentration inside the bag was 5 times higher than that outside the bag. The content of the bag was analysed for activity which was found to be close to values obtained when ferricyanide in a 4:1 molar ratio was added to hemolysate (Prep. 1).

TABLE III

THE EFFECT OF DIFFERENT CONCENTRATIONS OF FERRICYANIDE IN THE PREPARATION OF "FERRICYANIDE–FERRIHEMOGLOBIN"

"Ferricyanide-ferrihemoglobin" was prepared as described under Prep. 2, except that the concentration of $K_3Fe(CN)_6$ was varied as indicated below. Activity was determined by Assay 2. Ferrihemoglobin was determined by the method of EVELYN AND MALLOY[6].

Expt. No.	*Amounts (μmoles) mixed before gel filtration*		*Ferrihemoglobin formed (% of total pigment)*	*Ferrihemoglobin reduction (μmoles/min per mg protein × 10⁵)*
	Hemoglobin in hemolysate	*Potassium ferricyanide*		
1	1.0	1.0	26	5.6
2	1.0	2.0	53	36.4
3	1.0	4.0	95	193.0
4	1.0	15.0	98	200.0
5	1.0	45.0	98	212.0

The binding of ferrocyanide could also be demonstrated directly. Heating "ferricyanide hemolysate" diluted 1:1 with 14% (w/v) trichloroacetic acid at 100° for 30 min released ferrocyanide in an approximately 4:1 molar ratio—from its binding to ferrihemoglobin. The ferrocyanide could be determined quantitatively in the clear supernatant by the method of AVRON AND SHAVIT[2]. Ferricyanide was found only in trace amounts. The omission of heating resulted in only partial recoveries and confirmed the existence of strong forces holding the complex together.

Continuous absorption spectra of "Dowex hemolysates", to which ferrocyanide in different molar ratios was added were compared. The experiments were performed under aerobic and anaerobic conditions at pH 5.2 or 7.0. Apart from small differences, no spectral evidence for the binding of ferrocyanide to ferrihemoglobin could be found.

No difference between ferrihemoglobin and ferrihemoglobin–ferrocyanide could be demonstrated by comparing sedimentation rates in the analytical ultracentrifuge.

Other salts replacing potassium ferrocyanide as possible activators of ferrihemoglobin reduction

It was of interest to find out whether the effect of ferrocyanide in ferrihemoglobin reduction was specific or whether other substances might share this property. Sodium ferrocyanide had almost the same effect as potassium ferrocyanide. $FeSO_4$ was 10 times less effective while sodium nitroprusside was 50 times less effective. KCl, K_2SO_4, potassium cobaltichromate and potassium cobaltocyanide were without effect. These results indicate a rather high specificity for ferrocyanide ion.

The properties of the ferrihemoglobin–ferrocyanide system

Table IV shows a component study. It is evident that nicotinamide had no effect, while the influence of EDTA on the rate of reduction was weak. These two components were added routinely to the reaction mixture to prevent artifacts due to heavy metals and pyridine nucleotidase. The reaction was completely dependent on the addition of NADH. Of particular interest was the observation that in the presence of ferrocyanide the system was specific for NADH, NADPH being inactive as an electron donor. Removal of ferrocyanide resulted in a ferrihemoglobin preparation which was inactive.

TABLE IV

COMPONENT STUDY OF THE FERRIHEMOGLOBIN REDUCTION SYSTEM

Assay 2 was employed. "Ferricyanide hemolysate" (Prep. 2) was used. Specific activity of control was 300×10^{-5}.

Component omitted	*Ferrihemoglobin reduction (% of control)*
None (complete system)	100
Nicotinamide	100
EDTA (disodium salt)	80
NADH	0.0
NADPH instead of NADH	0.0
$K_4Fe(CN)_6$ removed by Dowex treatment)	0.1

An exponential dependence of the rate of reduction on the concentration of hemolysate was observed. The reaction mixture containing ferrihemoglobin in a concentration of 0.025 μmole/ml and causing a change in absorbance of about 0.500 per 5 min was found to be the most convenient for spectrophotometric measurements and was therefore used as standard in Assay 2.

The influence of NADH concentrations on the system was also studied. No sharp changes in rate were obtained between $1 \cdot 10^{-3}$ and $2 \cdot 10^{-5}$ M.

Fig. 2 illustrates the pH dependence of the system. It is evident that the pH optimum was around 5.2. The preparations were quite stable. Using Assay 2, it was found that hemolysates kept at 4° were active for at least 8 days.

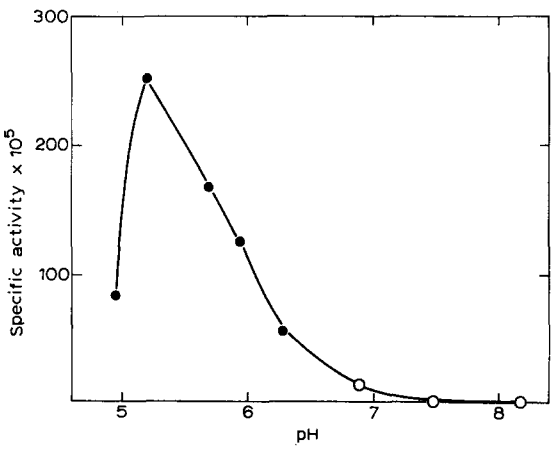

Fig. 2. The effect of pH on ferrihemoglobin reduction in "ferricyanide hemolysates". Assay 2 was employed except for buffer: solid symbols, citrate buffer; open symbols, Tris–HCl buffer. The pH was determined in the reaction mixture after the reaction was completed.

DISCUSSION

VESTLING[21] found that ferrihemoglobin formed with nitrite is reduced much more slowly and less completely by ascorbic acid at pH 7.0 and 0° than the ferri-

hemoglobin formed with ferricyanide. This was assumed to be due to a difference of the two ferrihemoglobins, although, as far as their spectrum was concerned, they were found quite similar[1]. Doubt was cast on this explanation by GIBSON[7], since the addition of ferrocyanide to the nitrite-ferrihemoglobin-ascorbic acid system increased the rate of reduction to that found for ferricyanide-ferrihemoglobin. GIBSON presented evidence that ferrocyanide does not act as a carrier but could not explain the nature of this catalysis.

In this paper it was demonstrated that by oxidizing hemolysate with ferricyanide, preparations are obtained in which the enzymatic reduction of ferrihemoglobin by NADH proceeds at by far the highest rate reported to date. The effect of ferricyanide was shown to be 2-fold: (a) oxidation of hemoglobin to ferrihemoglobin; (b) binding of ferrocyanide to ferrihemoglobin producing a complex responsible for the rate of ferrihemoglobin reduction. When the bound ferrocyanide was removed from ferrihemoglobin by Dowex treatment (Table II, 6) the hemolysates became similar to those prepared by nitrite as far as ferrihemoglobin reduction was concerned.

The simplest explanation of the data would be to suggest that ferrocyanide acts as an electron carrier, being alternately oxidized and reduced according to the following reactions:

$$\text{Ferrihemoglobin} + 4 \text{ ferrocyanide} \rightarrow \text{ferrohemoglobin} + 4 \text{ ferricyanide} \quad (1)$$

$$4 \text{ Ferricyanide} + 2 \text{ NADH} \rightarrow 4 \text{ ferrocyanide} + 2 \text{ NAD}^+ + 2 \text{ H}^+ \quad (2)$$

However, several lines of evidence strongly argue against such an hypothesis:

(a) As GIBSON[7] pointed out for the non-enzymatic reduction of ferrihemoglobin by ascorbic acid, in order that the first reaction proceeds from left to right, the ratio ferri-/ferrocyanide must be less than 1:27 000. Thus, the proceeding of the reaction to the right is very improbable. One may argue that oxygen, by combining with ferrohemoglobin, could so alter the value of the ferrihemoglobin/ferrohemoglobin quotient that the effective electromotive force may nevertheless allow the reaction to proceed to the right. However, it was shown that the rate of the ferrocyanide-activated reduction of ferrihemoglobin was the same under aerobic and anaerobic conditions (see Figs. 1A and B).

(b) Electron carriers usually act at concentrations which are many times lower than the concentrations of their substrate. However, ferrocyanide acted at concentrations which were of the same order of magnitude as those of the substrate. As shown in Fig. 1, ferrocyanide demonstrated its optimal catalytic effect at a definite molar ratio of 4:1 to ferrihemoglobin. This relationship between a carrier and the final electron acceptor is unexpected. It is interpreted as indicating that ferrocyanide acts by binding to the ferrihemoglobin molecule (1 ferrocyanide for each heme moiety), and not by serving as an electron carrier.

(c) Ferricyanide is reduced by both NADH and NADPH in the presence of a highly purified "ferrihemoglobin reductase" isolated from erythrocytes[24], according to Eqn. 2 (see above). Therefore, if the ferrocyanide generated in this reaction were acting as a carrier, it should be able to reduce ferrihemoglobin with NADH or NADPH. However, an absolute specificity of the reaction for NADH was demonstrated (Table IV). This fact suggests that ferrihemoglobin is not reduced by ferrocyanide but by a factor (enzyme) bound to or reacting specifically with NADH.

These considerations lead us to consider the carrier hypothesis as being extremely unlikely. The binding to ferrihemoglobin is thought to result in structural changes in the ferrihemoglobin molecule, these changes being responsible for the activation of the reducing system. The activating effect of ferrocyanide on the non-enzymatic reduction of ferrihemoglobin by ascorbic acid[7] can be interpreted in a similar way. Whether or not such activation represents a model for an activating reaction *in vivo* remains to be investigated.

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