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Evidence for the extracellular reduction of ferricyanide by rat liver

A trans-plasma membrane redox system

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1. Reduction of ferricyanide by the isolated perfused rat liver and by isolated rat hepatocytes was studied. 2. Ferricyanide was reduced to ferrocyanide by the perfused liver at a linear rate of 0.22 μmol/min per g of liver. Ferricyanide was not taken up by the liver and the perfusate concentration of ferricyanide + ferrocyanide remained constant throughout the perfusion. Perfusate samples from livers perfused without ferricyanide did not reduce ferricyanide. 3. Isolated hepatocytes reduced ferricyanide in a biphasic manner. The initial rate of 2.3 μmol/min per g of cells proceeded for approx. 3 min and derived from low-affinity sites (apparent $K_m > 1.3$ mM). The secondary rate of 0.29 μmol/min per g of cells was maintained for the remainder of the incubation and derived from higher affinity sites (apparent K_m 0.13 mM). Disruption of the cells resulted in an increase in the low-affinity rate and a decrease in the high-affinity rate. 4. Ferrocyanide was oxidized by isolated hepatocytes but not by perfused liver. The apparent K_m for ferrocyanide oxidation by hepatocytes was 1.3 mM. 5. Oxidized cytochrome *c* was reduced by isolated hepatocytes in the presence of 1 mM-KCN but at a rate less than that of the reduction of ferricyanide. 6. Properties of the ferricyanide-reducing activities of intact hepatocytes and the perfused liver were examined. The low-affinity rate, present only in cell and broken cell preparations, was inhibited by 1 μM-rotenone and 0.5 mM-ferrocyanide, and stimulated by 0.1 mM-KCN. The mitochondrial substrate, succinate, also stimulated this rate. The perfused liver showed only a high-affinity activity for ferricyanide reduction. This activity was also present in liver cells and was unaffected by rotenone, antimycin A, KCN, NaN_3, or *p*-hydroxymercuribenzoate but was inhibited by 2.6 mM-$CaCl_2$, 2-heptyl-4-hydroxyquinoline-*N*-oxide and ferrocyanide. Overall, these results are consistent with the occurrence of a trans-plasma membrane redox system of liver that reduces extracellular ferricyanide to ferrocyanide. The reduction process shows properties which are similar to that of the NADH:ferricyanide oxidoreductase found in isolated liver plasma membranes but different from that of mitochondria.

Manyai & Szekely (1954) first observed that extracellular ferricyanide when added to the medium of erythrocyte suspensions induced ATP synthesis inside the cells. Since ferricyanide, an anionic species carrying three negative charges, could not penetrate into the cells (Szekely *et al.*, 1952), it was concluded that the oxidizing agent effected ATP synthesis without direct contact with the glycolytic system. Subsequently Passow (1963) confirmed and extended the findings of Manyai & Szekely (1954) to show that ferrocyanide, in contrast to ferricyanide, induced ATP breakdown. Confirmation of the reduction of extracellular ferricyanide by intact erythrocytes followed (Dormandy & Zarday, 1965) and it was proposed by Mishra & Passow (1969) that the reduction was carried out by a transmembrane transhydrogenase which transferred electrons from an internal redox donor to the

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external ferricyanide. Orringer & Roer (1979) noted that dehydroascorbate enhanced ferricyanide reduction by erythrocytes and on this basis proposed that ascorbate was involved in the transmembrane redox system, acting as a redox carrier to reduce ferricyanide on the outside of the cell.

Studies with isolated plasma membranes from various types of cells have indicated that NADH:ferricyanide reductase activity was present in these preparations (for example see reviews by Löw & Crane, 1978, and Crane *et al.*, 1979). Part of this activity was attributed to the NADH:cytochrome b_5 reductase (Strittmatter & Velick, 1956) which has also been found associated with many plasma-membrane preparations (Kilberg & Christensen, 1979; Jarasch *et al.*, 1979). Based on studies of NADH:cytochrome *c* reductase activity in endomembranes (De Pierre & Ernster, 1977) it might be expected that this activity was exclusively located on the inner surface of the plasma membrane. In this regard, Kant & Steck (1973) have shown that in erythrocyte membranes the NADH:cytochrome *c* reductase activity was located predominantly on the cytoplasmic side of the membrane. On this basis it appears unlikely that the NADH:cytochrome *c* reductase can act as the transmembrane electron carrier.

An enzyme that might account for the observed transmembrane reduction of ferricyanide by erythrocytes is the NADH oxidoreductase which has been purified from erythrocyte membranes and which acts only as a ferricyanide reductase (Wang & Alanpovic, 1978). In erythrocyte membranes it has also been shown that open ghosts have more NADH:ferricyanide reductase activity than do closed vesicles with the exclusive exposure of the cytoplasmic side. This was consistent with a transmembrane NADH:ferricyanide reductase which oxidized internal NADH by reduction of external ferricyanide (Mackellar *et al.*, 1979; Löw *et al.*, 1980).

In the present paper we present evidence for the extracellular reduction of ferricyanide by perfused liver and isolated rat hepatocytes. Some of the properties of this trans-plasma membrane redox system are also described.

Materials and methods

Materials

Sodium ferro[^{14}C]cyanide was purchased from New England Nuclear. [^{3}H]Inulin, 3-*O*-methyl-D-[1-^{3}H]glucose, and ^3H_2O were purchased from The Radiochemical Centre, Amersham. Rotenone, 2-heptyl-4-hydroxyquinoline-*N*-oxide, inulin, cytochrome *c*, *p*-hydroxymercuribenzoate, and antimycin A (type III) were purchased from Sigma. Potassium ferricyanide, potassium ferrocyanide, ammonium persulphate and KCN were analytical grade reagents from BDH. Potassium ferricyanide was converted to sodium ferricyanide by passage through a column of cation-exchange resin. NaN_3 was from Merck.

Hepatocyte isolation and incubation

Isolated hepatocytes were prepared from fed rats (180–220g) essentially as described by Berry & Friend (1969), with the omission of hyaluronidase. Buffers were equilibrated with O_2/CO_2 (19:1) before use.

Incubations were conducted at 37°C with shaking (90 oscillations/min) in stoppered vials of 20ml capacity. Incubations contained 1.3mM-$CaCl_2$, 0.5mM-sodium ferricyanide and approx. 30mg wet wt. of cells in a total volume of 6ml of Krebs–Henseleit buffer, pH7.4 (Dawson, 1969) equilibrated with O_2/CO_2 (19:1). Samples (1.5ml) were removed at various times (usually 2 and 20min) and placed in centrifuge tubes, precooled in an ice/water mixture. After 30min at 0°C, the tubes were centrifuged (8000*g* for 2min) and the concentration of ferricyanide was determined by measuring the A_{420} ($\varepsilon = 1000\,M \cdot cm^{-1}$).

Liver perfusion

Livers of fed rats were perfused at 37°C with 125ml of Krebs–Henseleit buffer (maintained at pH7.4) containing 1.3mM-$CaCl_2$ and 1.0 or 0.5mM-sodium ferricyanide and which was equilibrated with O_2/CO_2 (19:1). After 5min of non-recirculating perfusion to remove blood cells, the perfusion was changed to a recirculating mode. The concentration of ferricyanide was determined by monitoring the decrease in A_{420} of the perfusate and by determining the rate of ferrocyanide formation (Avron & Shavit, 1963). The mean of the two methods was used to calculate the rate of ferricyanide reduction. Agreement between the two methods was within 10%. To minimize reduction of ferricyanide in the absence of the liver, the apparatus was cleaned with 0.01M-NaOH between perfusions.

Results

Initial experiments were conducted to assess whether ferricyanide entered the liver cells to be reduced to ferrocyanide. Table 1 shows the results of these studies where the liver content (or estimated space) of radioactivity was determined after a 15min perfusion of the liver with medium containing 0.5mM-sodium ferri[^{14}C]cyanide. Similar perfusions using ^3H_2O, [^{3}H]inulin or 3-*O*-methyl-D-[1-^{3}H]glucose were conducted for comparison. Analysis of the

Table 1. *Estimation of ferricyanide space in isolated perfused rat liver*

Livers of fed rats were perfused as described in the Materials and methods section. Additions were made as shown. After 15 min of recirculating perfusion, the liver was quickly removed, blotted and freeze-clamped. Liver was powdered after freezing in liquid N_2, weighed out and homogenized in water (10 parts by vol). For inulin, the homogenate was heated at 100°C for 5 min to ensure solubilization. Homogenates were centrifuged (8000 *g* for 2 min) and a portion of the supernatant was counted for radioactivity. Radioactivity content of the perfusate was also determined at the beginning and the end of the perfusion. Average perfusate content of radioactivity was used to calculate the occupied space for each of the additions. Means ± S.E.M. are shown with the number of perfusions in parentheses.

Additions	Estimated space (μl/g wet wt. of liver)
^3H_2O	444 ± 9 (3)
[^{3}H]Inulin (1 mg/ml)	92 ± 3 (3)
3-*O*-Methyl-D-[1-^{3}H]glucose	451 (2)
Potassium ferri[^{14}C]cyanide (1 mM)	86 ± 6 (3)

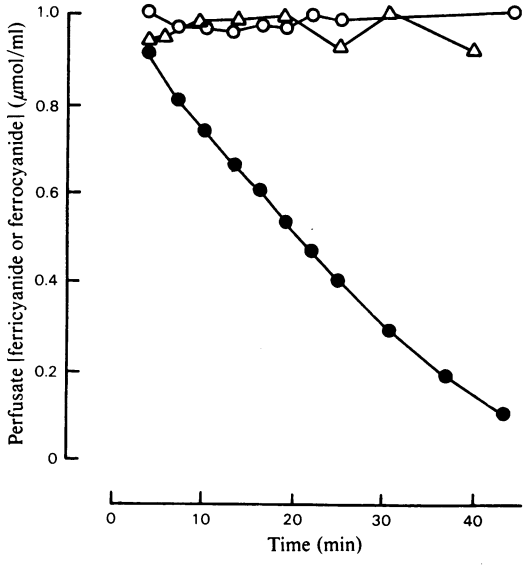

Fig. 1. *Reduction of ferricyanide by isolated perfused rat liver*

Livers of fed rats were perfused as described in the Materials and methods section. The concentration of ferricyanide (●) was determined by monitoring the decrease in A_{420} of the perfusate. The total ferricyanide + ferrocyanide (○) was determined by prior addition of excess ammonium persulphate to the perfusate samples before measuring A_{420}. Control perfusions were also conducted, omitting ferricyanide from the perfusate (△); ferricyanide-reducing substances were determined by adding 2 ml samples of perfusate to 24 μl of 100 mM-potassium ferricyanide in a cuvette at 25°C. These were allowed to stand for 10 min before the A_{420} was determined. Representative data from nine perfusions (three each) are shown.

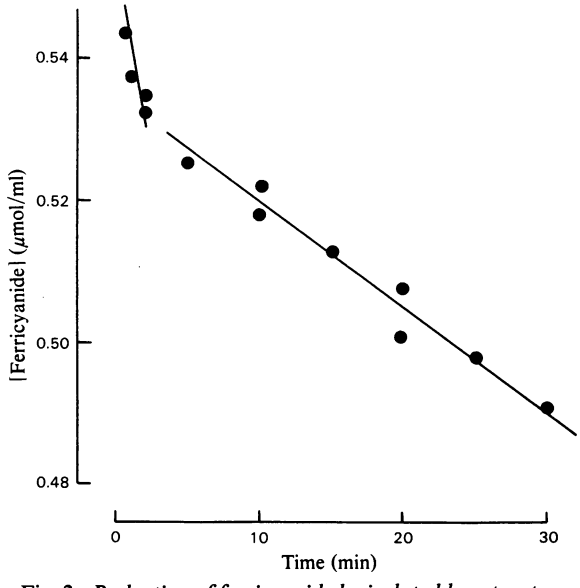

Fig. 2. *Reduction of ferricyanide by isolated hepatocytes*

Hepatocytes were prepared from fed rats and incubated as described in the Materials and methods section. Representative data from 17 liver cell preparations are shown.

radioactivity content of the livers indicated that inulin and ferricyanide spaces were similar (approx. 90 μl/g wet wt. of liver) and considerably less than either that of methylglucose or water. The estimated space for water was less than that predicted by determining the dry weight of fresh liver [718 ± 7 ($n = 3$) μl/g wet wt.], and probably indicated that insufficient time was allowed for equilibrium to be attained. Perfusion times of 15 min were chosen, as longer times gave rise to substantial conversion of ferricyanide to ferrocyanide. Nevertheless, the data suggest that neither ferricyanide nor ferrocyanide entered the liver cells, consistent with similar conclusions by other workers using erythrocytes (Szekely *et al.*, 1952).

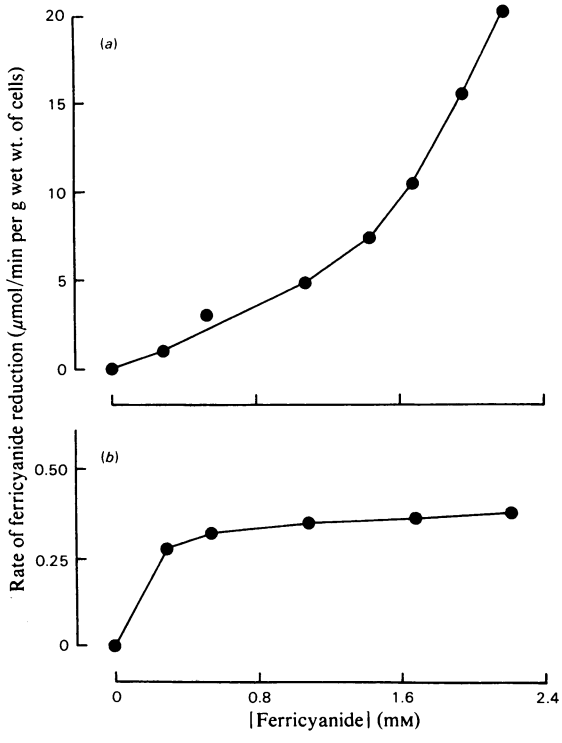

Fig. 3. *Effect of ferricyanide concentration on the rate of ferricyanide reduction by isolated hepatocytes*
Hepatocytes were prepared and incubated as described in the Materials and methods section. The initial rapid rate of ferricyanide reduction (see Fig. 2) was determined by monitoring the absorbance change over the initial 2 min of incubation (*a*); the second slower rate of ferricyanide reduction was determined over the subsequent 18 min (*b*).

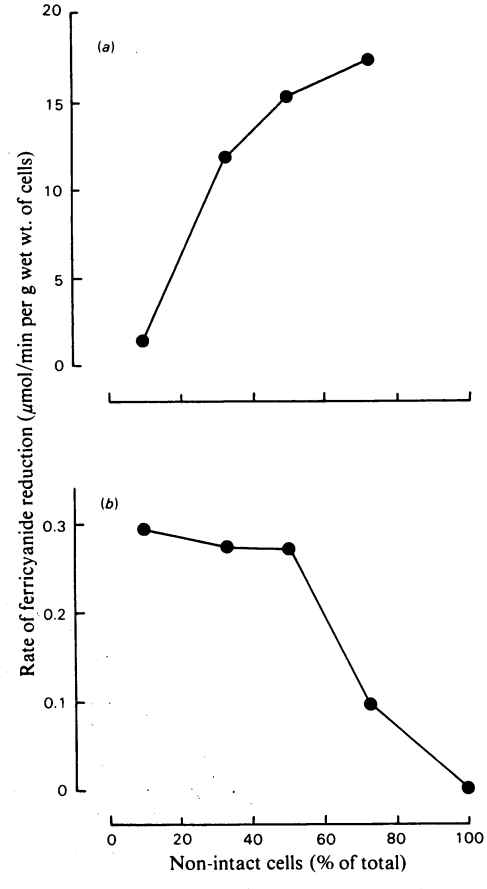

Fig. 4. *Effect of cellular integrity on the rate of ferricyanide reduction by isolated hepatocytes*
Hepatocytes were prepared as described in the Materials and methods section and divided into five portions. One portion remained untreated; each of the other four portions was treated to cause varying degrees of cell rupture by homogenization with a Dounce homogenizer. The proportion of non-intact cells (% of total) was assessed by determining the proportion of the total lactate dehydrogenase that was present in the supernatant following centrifugation of the cell suspension (150 ***g*** for 75 s). Total activity in cells plus medium was measured in each cell suspension diluted 10–20-fold in the presence of 0.5% Triton X-100. Lactate dehydrogenase activity was determined by the method of Kornberg (1955). The low-affinity (*a*) and high-affinity (*b*) ferricyanide-reducing activities were determined for each cell suspension as described in the Materials and methods section.

Fig. 1 shows that 1 mM-ferricyanide was reduced to ferrocyanide by recirculating perfusion through the isolated rat liver. Since the total perfusate concentration of ferricyanide + ferrocyanide remained constant throughout the perfusion it appeared unlikely that ferricyanide entered the cell to be catabolized to other products.

Possible adverse effects of ferricyanide on liver integrity was assessed. Leakage of lactate dehydrogenase was determined (Kornberg, 1955) for both control and ferricyanide-perfused livers. Lactate dehydrogenase activity in the perfusate at 20 min was 7.20 and 8.07 nmol/min per ml (25°C) respectively, for control and ferricyanide perfusions. This represented less than 0.02% of the liver content of lactate dehydrogenase activity.

To test whether the reduction of ferricyanide by the perfused liver resulted from reducing substances synthesized by the liver and released into the perfusate, a control perfusion (no ferricyanide) was conducted in which perfusate samples were taken at various times during a 40 min perfusion and incubated at 25°C for 10 min with 1 mM-ferricyanide. Fig. 1 shows that no reduction of ferricyanide occurred unless ferricyanide was directly in contact with the liver.

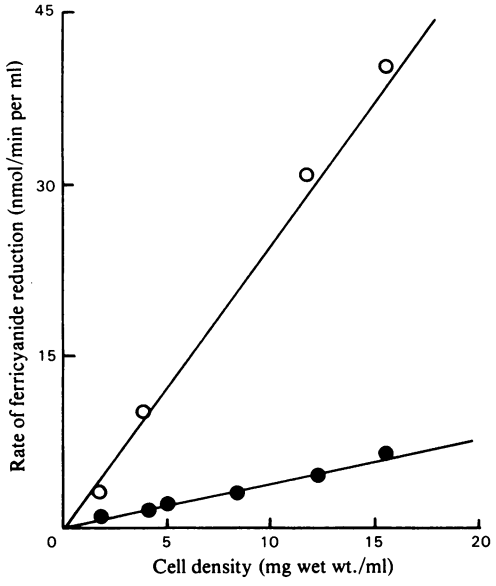

Fig. 5. *Effect of cell density on the rate of ferricyanide reduction by isolated hepatocytes*

Hepatocytes were prepared and incubated as described in the Materials and methods section. The initial rapid rate [low-affinity rate, (○)] of ferricyanide reduction was determined by monitoring the absorbance change at 30s intervals for the initial 2min of incubation. The second lower rate of ferricyanide reduction [high-affinity rate, (●)] was monitored over the subsequent 18min. No correction for overlap of the two rates has been made.

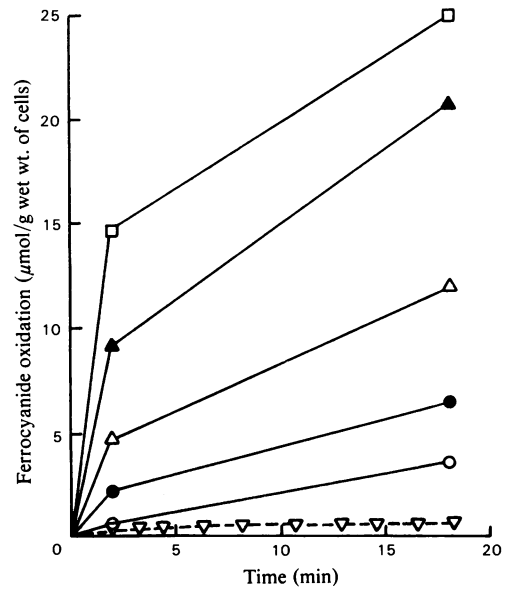

Fig. 6. *Ferrocyanide oxidation by isolated hepatocytes and perfused liver*

Hepatocytes were prepared from fed rats as described in the Materials and methods section. Incubations contained 1.3mM-$CaCl_2$, approx. 30mg wet wt. of cells and the amounts shown of ferrocyanide in a total volume of 6ml of Krebs–Henseleit bicarbonate buffer equilibrated with O_2/CO_2 (19:1). Samples (1.5ml) were removed at the times indicated and placed in centrifuge tubes in an ice/water mixture. After 30min at 0°C the tubes were centrifuged (8000*g* for 2min) and the A_{420} of the supernatant solution was measured. Initial concentrations of ferrocyanide in hepatocyte incubations were 0.08mM (○), 0.25mM (●), 0.5mM (△), 1.0mM (▲) and 2.0mM (□). Livers of fed rats were perfused as described in the Materials and methods section. The initial perfusate ferrocyanide concentration was 0.5mM (broken line, ▽).

Because of the convenience of isolated hepatocyte suspensions for metabolic studies and also because cell types other than hepatocytes are eliminated, isolated hepatocytes were tested for their ability to reduce ferricyanide. Fig. 2 shows that two rates of reduction occurred. An initial rapid rate of 2.3μmol/min per g wet wt. of cells, of approx. 3min duration, was followed by a sustained slower rate of 0.29μmol/min per g wet wt. of cells. Each of these rates was affected differently by varying the concentration of ferricyanide (Fig. 3). Whereas the slow sustained rate showed hyperbolic saturation kinetics, the fast initial rate increased in a sigmoid manner with increasing ferricyanide concentrations. Estimates of the apparent K_m for ferricyanide for each rate also showed marked differences. Reciprocal plots of the slow rate as a function of the ferricyanide concentration indicated the apparent K_m to be 0.13mM; the apparent K_m for the fast initial rate could not be accurately determined but was at least an order of magnitude greater.

On average, cell populations were 90% intact as indicated both by microscopic appearance and by the proportion of lactate dehydrogenase found in the suspending medium. If the proportion of non-intact cells was increased by progressive damage with a Dounce homogenizer there was an increase in the low-affinity rate and a decrease in the high-affinity rate (Fig. 4).

Fully ruptured cells showed no high-affinity ferricyanide-reducing activity.

Fig. 5 shows that each of the high-affinity (slow) and low-affinity (fast) rates were proportional to the cell density.

Having observed that ferricyanide was reduced both by the isolated perfused liver and by hepatocytes it was of interest to assess whether ferrocyanide was oxidized by these systems. Whereas perfused liver did not oxidize ferrocyanide, rates of

oxidation by hepatocytes were significant (Fig. 6). The data of Fig. 6 were used to estimate the affinity of ferrocyanide for the oxidizing system; reciprocal plots indicated the apparent K_m to be approx. 1.3 mM. The data of Fig. 6 were also used to estimate the rate of re-oxidation of ferrocyanide that may occur during studies on the rates of ferricyanide reduction. Because of the relative low affinity of ferrocyanide for the oxidizing system, the back reaction involving re-oxidation of the formed ferrocyanide could contribute an error of no more than 10% to the rate of ferricyanide (0.5 mM) reduction. This could be minimized by using cell populations which contain as few as possible mitochondria (from broken cells), or eliminated by using the isolated perfused liver.

Reduction of oxidized cytochrome *c* was determined using isolated hepatocytes in the presence of 1 mM-KCN (Fig. 7). Even though a linear rate occurred, this rate was 0.031 μmol/min per g wet wt. of cells or approx. 10% of the high-affinity rate observed with 0.5 mM-ferricyanide (Table 2).

Effects of activators, inhibitors and substrates

An attempt to characterize the ferricyanide-reducing activities of the isolated hepatocytes and perfused liver was made and the effects of Ca^{2+}, activators, inhibitors and substrates were examined (Table 2). Ferricyanide was not reduced unless cells were present in the incubations. Similarly, if ferricyanide was omitted from the incubations there was no decrease in A_{420} of the incubation supernatants.

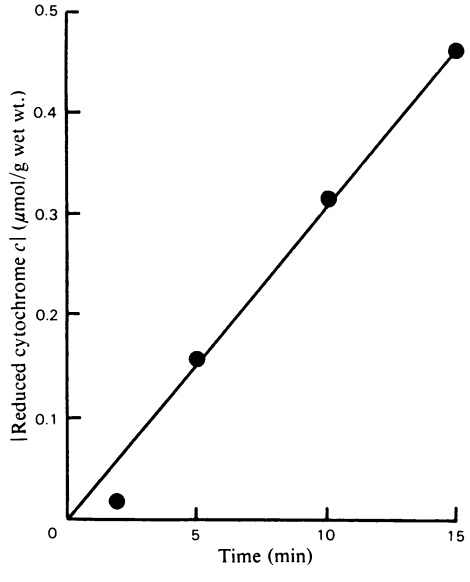

Fig. 7. *Reduction of oxidized cytochrome c by isolated hepatocytes*

Hepatocytes were prepared from fed rats as described in the Materials and methods section. Incubations contained 1.3 mM-$CaCl_2$, approx. 30 mg wet wt. of cells, 0.05 mM-cytochrome *c* and 1 mM-KCN in a total volume of 6 ml of Krebs–Henseleit bicarbonate buffer equilibrated with O_2/CO_2 (19 : 1). A_{550} was monitored.

The low-affinity rate was inhibited by 1 μM-rotenone and 0.5 mM-ferrocyanide and activated by 0.1 mM-KCN. Antimycin A (7.3 μM), 1 μM-2-heptyl-4-hydroxyquinoline-*N*-oxide, 0.5 mM-NaN_3 and high concentrations of Ca^{2+} (2.6 mM) each inhibited the low-affinity rate when compared with the control rate for each cell preparation. However these effects were not statistically significant. Inhibition by rotenone and stimulation by cyanide suggested that mitochondria from the small content of broken hepatocytes were responsible for the low-affinity rate of ferricyanide reduction, an activity which was undetectable using the isolated perfused liver (see below).

Table 2 also shows the effects of the above compounds on the high-affinity slow activity for hepatocytes and for the isolated perfused liver. This rate was unaffected by rotenone, antimycin A, azide, cyanide, or *p*-hydroxymercuribenzoate. 2-Heptyl-4-hydroxyquinoline-*N*-oxide (1 μM) and ferrocyanide (0.5 mM) each significantly inhibited the high-affinity rate, the latter possibly in a competitive manner. Omission of Ca^{2+} had no effect on the rate of ferricyanide reduction by the isolated perfused liver or hepatocytes. However, the rate was significantly inhibited in both preparations by 2.6 mM-$CaCl_2$. The mitochondrial substrate, succinate, markedly increased the rate of ferricyanide reduction with cells but was essentially without effect in the perfused liver (Table 2). These results were consistent with succinate-mediated stimulation of mitochondrial ferricyanide reduction where access to mitochondria was only possible when broken cells were present. When mitochondrial substrates were added to the cell preparation the high initial rate extended beyond the initial 2 min (see Fig. 2) and made it impractical to assess the high-affinity activity.

Relative effectiveness of redox acceptors

Comparison of the relative rates of ferricyanide, cytochrome *c* and oxygen reduction by intact and broken cells was made (Table 3). Oxygen and ferricyanide (high affinity) reduction by intact cells was greater than by broken cells. Reduction of ferricyanide (low affinity) or oxidized cytochrome *c* was greatest in broken cell preparations. The high-affinity rate of ferricyanide reduction, indicated above to be transmembranous, was undetectable in broken cell preparations.

Table 2. *Effects of inhibitors and activators on low- and high-affinity ferricyanide reduction activities of isolated hepatocytes*

Hepatocytes were prepared and incubated as described in the Materials and methods section. The ferricyanide concentration was 0.5 mM. The initial rapid rate (low affinity) of ferricyanide reduction was determined over the first 2 min of incubation. The second slower rate of ferricyanide reduction (high affinity) was monitored over the subsequent 18 min. No correction for overlap of the two rates has been made. Perfusion details were as described in the text. Where appropriate, means ± S.E.M. have been calculated and the number of cell preparations are given in parentheses. Student's *t* test was applied to assess the significance of the experimental situation versus the relevant control; *, $P < 0.05$; n.s., not significant.

	Ferricyanide reduction rate (μmol/min per g wet wt. of liver)		
	Hepatocytes		
Incubation	Low-affinity activity	High-affinity activity	Perfused liver
Control (1.3 mM-$CaCl_2$)	2.26 ± 0.25 (10)	0.28 ± 0.03 (11)	0.22 ± 0.006 (34)
Omissions			
Ferricyanide	0 (4)*	0 (4)*	0 (1)
Cells or liver	0 (5)*	0 (5)*	0 (2)
$CaCl_2$	3.20 ± 0.04 (3) n.s.	0.35 ± 0.05 (3) n.s.	0.20 ± 0.006 (4) n.s.
Additions (final concn.)			
$CaCl_2$ (2.6 mM)	1.46 ± 0.36 (3) n.s.	0.18 ± 0.02 (3)*	0.16 ± 0.007 (3)*
Rotenone (1 μM)	1.11 ± 0.34 (9)*	0.25 ± 0.03 (9) n.s.	0.19 ± 0.01 (3) n.s.
Antimycin A (7.3 μM)	1.56 ± 0.58 (4) n.s.	0.26 ± 0.04 (6) n.s.	0.18 ± 0.01 (3) n.s.
2-Heptyl-4-hydroxyquinoline-*N*-oxide (1 μM)	1.70 ± 0.73 (4) n.s.	0.23 ± 0.05 (4) n.s.	0.16 ± 0.004 (3)*
KCN (0.1 mM)	3.76 ± 0.69 (3)*	0.34 ± 0.02 (3) n.s.	0.21 ± 0.003 (3) n.s.
NaN_3 (0.5 mM)	1.89 ± 0.51 (7) n.s.	0.29 ± 0.05 (6) n.s.	0.18 ± 0.003 (3)
p-Hydroxymercuribenzoate (1 μM)	3.02 ± 0.69 (3) n.s.	0.25 ± 0.07 (3) n.s.	0.18 ± 0.02 (3) n.s.
Ferrocyanide (0.5 mM)	1.04 ± 0.52 (3)*	0.14 ± 0.04 (3)*	0.14 ± 0.01 (3)*
Succinate (10 mM)	4.24 ± 1.50 (3)*		0.19 ± 0.02 (3) n.s.

Table 3. *Comparison of the relative rates of ferricyanide, cytochrome c and oxygen reduction by intact and broken hepatocytes*

Hepatocytes were prepared and incubated as described in the Materials and methods section. Broken cells were prepared as described in the text. Incubations contained 0.5 mM-ferricyanide, 0.05 mM-cytochrome *c* (with 1 mM-KCN) and were equilibrated with O_2/CO_2 (19:1). Oxygen uptake was determined polarographically. Mean values are shown from triplicate determinations from at least two cell preparations.

	Rate of acceptor reduction (μmol/min per g wet wt. of cells)	
Acceptor	Cells	Broken cells
Ferricyanide (low affinity)	2.30	18.00
Ferricyanide (high affinity)	0.29	
Oxidized cytochrome *c*	0.03	0.54
Oxygen	1.67	0.49

Discussion

Evidence is presented for a ferricyanide reducing activity in intact liver. These findings are consistent with the concept of a trans-plasma membrane electron transport system in which the enzyme can react with the artificial non-permeable electron acceptor, ferricyanide, but shows very little activity with cytochrome *c*. Reduction does not appear to be accomplished by movement of a reducing agent, such as glutathione or other reductants, through the membrane since cytochrome *c* reduction did not occur. In addition, perfusate samples from perfusions not containing ferricyanide did not contain ferricyanide-reducing substances. Thus a direct interaction of ferricyanide with enzymes of the plasma membrane electron transport system appears likely. The lack of inhibition by *p*-hydroxymercuribenzoate, which has poor permeability in membranes, indicates that a thiol compound cannot be the transmembrane carrier. Since ferricyanide (0.1–2.4 mM) did not affect lactate dehydrogenase leakage by the liver and did not inhibit gluconeogenesis from 1.3 mM-lactate (M. G. Clark & G. S. Patten, unpublished work), it appeared unlikely that the permeability properties of the plasma membrane and the integrity of the liver were impaired by the presence of ferricyanide.

Intact liver showed only a high-affinity ferricyanide-reducing activity (apparent K_m 0.13 mM-ferricyanide) which was unaffected by rotenone, antimycin A, cyanide, azide or the mitochondrial substrate, succinate.

Isolated hepatocyte suspensions, although considered to be sufficiently intact for most metabolic studies (e.g. see Krebs *et al.*, 1974), invariably contain a small percentage of non-intact cells. In the present studies, the non-intact cells contributed free mitochondria to the cell suspension. These mitochondria appeared to account for (*a*) the low-affinity rapid rate of ferricyanide reduction (apparent $K_m > 1.3$ mM; Fig. 2 and Table 2), and (*b*) the oxidation of ferrocyanide (Fig. 6) (apparent K_m 1.3 mM). Neither of these activities was detected using the perfused liver. The reduction by hepatocyte suspensions of oxidized cytochrome *c* in the presence of KCN (Fig. 7), usually regarded as a mitochondrial process, may have also resulted from free mitochondria in the preparation. The low-affinity ferricyanide-reducing activity of isolated hepatocytes was characterized by being inhibited by 1 μM-rotenone and by being stimulated by the mitochondrial substrate, succinate. In addition the low-affinity rapid rate was of short duration (less than 3 min), possibly reflecting exhaustion of mitochondrial reducing power, that could be increased by succinate or cyanide and decreased by high concentrations of ferricyanide.

The findings of Mishra & Passow (1969) and Dormandy & Zarday (1965) support the concept of a protonophoric transmembrane NADH dehydrogenase. They showed that reduction of ferricyanide by intact erythrocytes was accompanied by proton transfer across the membrane and possibly by ATP formation. In the present study the identity of the reductant is unknown. Whereas there was no direct evidence to support the view that NADH is in fact involved, a similarity was noted between the property of the presently described cell system for ferricyanide reduction and that of the isolated plasma membrane NADH:ferricyanide oxidoreductase. Thus the K_m for ferricyanide, which was 0.13 mM for the cell high affinity activity, approximated to that for the isolated erythrocyte plasma membrane activity (Zamudio *et al.*, 1969). However, azide, previously reported to inhibit the plasma membrane NADH:ferricyanide oxidoreductase (Ramasarma *et al.*, 1980), had no effect on the high-affinity activity of hepatocytes.

The natural electron acceptor for the transmembrane NADH dehydrogenase of erythrocytes has been considered previously (Löw *et al.*, 1979) but remains to be determined. The present findings do not rule out the possibility that oxygen may be an acceptor such that the oxygen consumed by this process would represent a component of the cyanide-insensitive respiration of liver (Gayda *et al.*, 1977). However, other acceptors should also be considered. The rapid electron transport of ferricyanide found in all isolated plasma membranes and of similar activity to that of mitochondria (Crane *et al.*, 1979) suggests that an iron compound may be the natural acceptor.

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