CXXVI. ANAEROBIC OXIDATIONS. ON FERRICYANIDE AS A REAGENT FOR THE MANOMETRIC INVESTIGATION OF DEHYDROGENASE SYSTEMS

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THE following paper presents a preliminary account of the use of ferricyanide as a reagent for the manometric study of dehydrogenase systems, and is intended to form an introduction to more complete studies on this subject. The principle of the new technique depends upon the fact that when 1 mol. ferricyanide is reduced, 1 mol. acid is formed and in bicarbonate media this gives rise to 1 mol. CO_2 which can be estimated manometrically. The relevant equations are:

$$H + Fe(CN)_6''' \rightarrow H^+ + Fe(CN)_6''''$$
$$H^+ + HCO_3' \rightarrow H_2CO_3 \rightarrow CO_2 + H_2O.$$

This method has been used already for the manometric estimation of glutathione and reduced cozymase, both of which are readily oxidized (in the absence of any enzyme) by ferricyanide [Haas, 1937].

It has been found that the technique can be profitably adopted for the study of certain aspects of oxidizing systems in tissue slices and extracts, and forms a valuable additional method for the investigation of the dehydrogenases.

Ferricyanide is, so far as we have investigated it, a relatively non-toxic substance, and indeed it has been shown by Mendel & Strelitz [1937] and by Orr & Stickland [1938] that its presence at a concentration of 0·01 *M* has but little effect on anaerobic glycolysis and on the respiration of tumour tissue. Ferrocyanide is also, at similar concentrations, relatively non-toxic and has the advantage over most reduced dyestuffs of being non-autoxidizable.

Technique. Manometric methods of Warburg have been adopted for following the rate of reduction of ferricyanide by enzyme systems. Our technique consists in placing within the main compartment of a Warburg manometer cup tissue slices immersed in, or tissue extracts mixed with, a saline $NaHCO_3$ (0·025 *M*) medium and in placing in the side tube 0·2 ml. of a ferricyanide-$NaHCO_3$ solution. The latter solution is made up by mixing 5 ml. 10 % $Na_3Fe(CN)_6$ with 1 ml. 0·16 *M* $NaHCO_3$. The vessel is now gassed with 95 % N_2 + 5 % CO_2 and after thermal equilibrium has been established, the ferricyanide solution is tipped from the side tube into the main vessel and the velocity of gas output is followed.

In the case of tissue slices, the medium consists of $NaHCO_3$ 0·025 *M*, KCl 0·006 *M*, $CaCl_2$ 0·001 *M*. The volume is made up to 3 ml., after allowance has been made for the addition of other desired substances with NaCl solution so that the final solution has an osmotic strength of approximately 0·16 *M*. Sticks of yellow P are placed within the cylindrical tube in the main vessel of the manometer cup to ensure anaerobic conditions when using tissue slices. This precaution is

unnecessary in the case of the tissue extracts we have employed and in the latter case also, KCl and $CaCl_2$ are omitted from the medium.

Tissue slices, cut from the freshly dissected organs of an animal, are usually between 0·2 mm. and 0·4 mm. thick and have a dry weight of approximately 10 mg.

The initial *p*H of the media containing tissue slices or extracts is 7·4 and the temperature at which the experiments are carried out is 38·4°.

The manometric determination of the rate of reduction of ferricyanide may be roughly checked by estimating the resultant ferrocyanide at the end of an experiment. This can be done by making use of the sensitive colour reaction which takes place between ferrocyanide, acetic acid and ammonium molybdate solution. To the solution (3 ml.) containing ferrocyanide are added 0·5 ml. glacial acetic acid and 1 ml. 10 % ammonium molybdate solution. The solution may be centrifuged after addition of acetic acid to remove protein and the ammonium molybdate solution then added. A deep brown coloration appears which may be compared with the colour produced by known amounts of ferrocyanide treated similarly with acetic acid and ammonium molybdate solution. A further check may be obtained, if this is required, by adding Fe^{+++} to the solution of ferrocyanide and estimating the prussian blue formed.

Reduction of ferricyanide by sodium succinate and by choline in presence of tissue slices. In the presence of intact rat liver and rat brain slices and of sodium succinate, ferricyanide undergoes a rapid reduction. A slow reduction takes place in the absence of added substrate, this being partly due to oxidation of the glutathione and ascorbic acid present in the tissue slices. Typical results are shown in Table I, the symbol $Q^{N_2}_{CO_2}$ representing the vol. CO_2 in μl. produced per hr. for 1 mg. dry wt. tissue. The reduction due to succinate is presumably to be attributed to the action of the succinic acid dehydrogenase present in the tissues.

Table I

Tissue	Substrate	$Q^{N_2}_{CO_2}$ (over 1st hr.)
Liver slices	None added	6·3
,,	Sodium succinate 0·01 *M*	49·8
,,	Choline chloride 0·012 *M*	48·9
Brain cortex slices	None added	4·1
,,	Sodium succinate 0·01 *M*	17·5
No tissue present	Sodium succinate 0·01 *M*	No gas output
	Choline chloride 0·012 *M*	Very little gas output (8 μl. 1 hr.)

Choline, in the presence of rat liver slices, also rapidly reduces ferricyanide. It has been shown by Mann & Quastel [1937] that choline is oxidized by an oxidase in rat liver to betaine aldehyde. The fact that choline is oxidized anaerobically by ferricyanide in the presence of rat liver indicates that the enzyme system in question consists partly of a dehydrogenase.

Reduction of ferricyanide by dehydrogenase-coenzyme systems in tissue slices. The addition of sodium lactate or sodium *l*-malate to tissue slices effects only a small increase in the rate of reduction of ferricyanide. The addition of cozymase[1] (4 mg. of a partially purified specimen) to the system however brings about a relatively rapid rate of reduction. Some preliminary results are shown in Table II. It will be seen that the presence of cozymase considerably enhances the rate of

[1] The cozymase used in this work was a specimen obtained after purification of a crude sample kindly supplied to us by Messrs Hoffmann la Roche, Basle.

Table II

Tissue slices (rat)	Substrate (Na salt)	$Q_{CO_2}^{N_2}$ In absence of added cozymase	$Q_{CO_2}^{N_2}$ Cozymase solution (0·2 ml.) added
Liver	None added	6·3	10·1
	dl-Lactate 0·026 *M*	10·7	17·6
	l-Malate 0·06 *M*	5·1	17·1
	dl-Glycerate 0·06 *M*	8·2	16·2
Brain cortex	None added	4·1	5·9
	dl-Lactate 0·026 *M*	5·0	11·5
	l-Malate 0·03 *M*	6·1	9·5
Diaphragm	None added	5·2	6·0
	dl-Lactate 0·026 *M*	6·2	12·7
Kidney	None added	12·1	18·6
	l (+) Glutamate 0·026 *M*	15·0	25·2

reduction of sodium ferricyanide by *dl*-lactate, *l*-malate and *dl*-glycerate in the presence of liver slices. The same phenomenon takes place with *dl*-lactate and *l*-malate in the presence of brain cortex slices. The accelerating action of cozymase can also be seen with diaphragm using *dl*-lactate and with kidney slices using *l* (+) glutamate.

These experiments show that the effects of added cozymase which have previously been studied only with tissue extracts, can be seen and studied in intact tissue slices.

We have found as yet no evidence that pyruvate in the absence of added catalysts can be oxidized by ferricyanide in the presence of tissue slices. The addition of cozymase does not accelerate the reduction of ferricyanide by pyruvate in the presence of brain cortex slices. It is doubtless for this reason that the rate of reduction of ferricyanide is small compared with the rate of O_2 uptake by lactate in the presence of tissue slices, for the latter rate also involves the oxidation of intermediaries such as pyruvate.

Reduction of ferricyanide by dehydrogenase systems in lysed red blood cells. Lysed red blood cells are comparatively rich sources of lactic and malic acid dehydrogenases. This fact is shown in the results quoted in Table III. In these

Table III

NaCN (0·03 *M*) present throughout

Exp.	Lysed red blood cells (1 ml.)	Substrates (Na salts)	Gas output in 1 hr., μl. Without added cozymase	Gas output in 1 hr., μl. With added cozymase (0·2 ml.)
1	Human (1/5)	—	71·6*	85·7*
	,,	*dl*-Lactate 0·013 *M*	93·6*	388·2*
2	,,	—	101	110
	,,	*l*-Malate 0·1 *M*	131·1	322·5
3	Ox (1/5)	—	—	110·4
	,,	Hydroxymalonate 0·08 *M*	—	122·6
	,,	*dl*-Lactate 0·13 *M*	—	209·4
	,,	*dl*-Lactate 0·13 *M* + hydroxymalonate 0·08 *M*	—	155·3
	,,	Malate 0·13 *M*	—	212·9
	,,	Malate 0·13 *M* + hydroxymalonate 0·08 *M*	—	221·9
4	,,	*dl*-Lactate 0·13 *M*	—	189·9
	,,	*dl*-Lactate 0·13 *M* + hydroxymalonate 0·08 *M*	—	124·1

* These figures refer to the gas output after 30 min.

experiments well washed red blood cells (human or ox) were lysed by mixing with 4 times their volume of distilled water. NaCN (0·03 *M*) was used throughout to provide a fixative for the ketonic acids produced by oxidation.

The results given in Table III show that when *dl*-lactate or *l*-malate is added to lysed red blood cells rapid reduction of ferricyanide takes place so long as cozymase has been added to the system. The fact that the lactic acid dehydrogenase is of a similar nature to that present in bacteria or tissues is shown by the inhibition effected by the presence of hydroxymalonate (Exp. 3 and 4). The latter substance was shown to effect a specific inhibition of lactic dehydrogenase [Quastel & Wooldridge, 1928; confirmed by Green & Brosteaux, 1936], and to inhibit the oxidation of lactic acid by brain [Jowett & Quastel, 1937]. Malic acid oxidation by ferricyanide is either slightly or not inhibited by hydroxymalonate at the concentration tried (Exp. 3), and this fact serves to indicate the separate natures of malic and lactic acid dehydrogenases in lysed blood cells.

Although it is evident from the ferricyanide reduction that lactic acid dehydrogenase exists in lysed red blood cells, the amount of O_2 consumed by such lysed cells in the presence of lactate, cozymase and a carrier such as methylene blue, is small. The result of an experiment on this point is given in Table IV. Cyanide was added to ensure favourable conditions for the O_2 absorption to take place [cf. Green & Brosteaux, 1936]. The feeble O_2 uptake, compared with the rapid gas output in presence of ferricyanide—taking into account the fact that the reduction of 1 mol. O_2 to water corresponds to that of 4 mol. ferricyanide—shows that the reduction of methylene blue by the lactic dehydrogenase system must be slow. The system, in fact, is comparable with the lactic dehydrogenase system present in a dialysed aqueous extract of skeletal muscle which also shows little or no reduction of methylene blue.

Table IV

	O_2 uptake in 30 min.
1. 1 ml. lysed human red blood cells (1/5) + phosphate buffer (0·067 *M*) + 0·2 ml. cozymase + NaCN (0·067 *M*) + methylene blue (1/15000)	6 μl.
2. Ditto with the addition of *dl*-lactate (0·13 *M*)	30 μl.
	CO_2 output in 30 min.
3. 1 ml. lysed human red blood cells (1/5) + bicarbonate (0·025 *M*) + 0·2 ml. cozymase + NaCN (0·067 *M*). N_2/CO_2	91 μl.*
4. Ditto with the addition of *dl*-lactate (0·13 *M*)	396 μl.*

* The values of CO_2 output are not materially affected by the presence of phosphorus in the manometer vessels introduced to ensure strictly anaerobic conditions.

Reduction of ferricyanide by dehydrogenase systems in dialysed aqueous extracts of skeletal muscle. A dialysed aqueous extract of the skeletal muscle of a rabbit is prepared according to the method of Green *et al.* [1937], i.e. by extracting the well minced tissue with ice cold water for 30 min., precipitating the extract with acetone, dissolving the precipitate in water and dialysing and centrifuging the solution. The clear supernatant fluid forms, according to the above authors, a good source of triose, triosephosphate and α-glycerophosphate mutases, i.e. enzymes which, in presence of cozymase, bring about the interaction of triose, etc. with such α-ketonic acids as pyruvic and oxaloacetic.

It may be shown by the ferricyanide technique that the solution in question is rich in lactic, malic, glyceric and α-glycerophosphoric acid dehydrogenases, and it becomes evident that there is no point in postulating the existence of special mutases involving these molecules [cf. Green *et. al.*, 1936].

Table V

NaCN, when present, is 0·067 *M*

Exp.	Muscle extract ml.	Substrates (Na salts)	Gas output (μl.) in 1 hr. Without cozymase added	Gas output (μl.) in 1 hr. With cozymase (0·2 ml.) added
1	1	NaCN	67·0	77·0
	1	NaCN + *dl*-lactate 0·13 *M*	84·9	305·6
2	1·5	NaCN + α-glycerophosphate 0·13 *M*	78·2	240·3
3	1·5	NaCN + *l*-malate 0·13 *M*	104·4	260·5
4	1·5	NaCN	—	115·6
	1·5	NaCN + *dl*-lactate 0·13 *M*	—	430·9
	1·5	NaCN + *dl*-glycerate 0·13 *M*	—	246·7
	1·5	NaCN + α-glycerophosphate 0·13 *M*	—	252·8
5	1·5	NaCN + *dl*-glycerate 0·13 *M*	83·6	221·3
6	Nil	*dl*-glyceraldehyde (0·018 *M*)	28·2	—
	1·5	Nil	29·2	33·8
	[1·5	*dl*-Glyceraldehyde. No ferricyanide added (0·018 *M*)	58·5	79·4]
	1·5	*dl*-Glyceraldehyde (0·018 *M*)	208·4	362·7
7	Nil	Hexosediphosphate (0·013 *M*)	39·6	—
	1·5	Nil	18	47·9
	[1·5	Hexosediphosphate (0·013 *M*). No ferricyanide added	88·3]	—
	1·5	Hexosediphosphate (0·013 *M*)	463·1	—
	1·5	Hexosediphosphate (0·013 *M*) + NaF 0·05 *M*	457·3	—
8	Nil	Hexosediphosphate (0·013 *M*)	21	—
	1·5	Nil	29·2	33·8
	[1·5	Hexosediphosphate (0·013 *M*). No ferricyanide added	110·7*	137·1*]
	1·5	Hexosediphosphate (0·013 *M*)	724*	779*
	1·5	Hexosediphosphate (0·003 *M*)	325*	357*
9	1·5	NaCN + *dl*-lactate	—	370
	1·5	NaCN + *dl*-lactate	—	112 (alkali-heated cozymase)

* Figures obtained after 30 min.

A dialysed muscle extract, prepared as above, brings about a rapid reduction of ferricyanide in the presence of lactate so long as cozymase is added to the system. NaCN should be also added: its favourable effect is presumably due to HCN-fixation of pyruvic acid which was shown by Quastel & Wooldridge [1928] to have a marked inhibitory action on the activation of lactate by bacterial lactic dehydrogenase. The inhibitory action of pyruvate was confirmed by Bernheim [1928] for the lactic dehydrogenase of yeast, by Birch & Mann [1934] and more recently by Green & Brosteaux [1936] for the lactic dehydrogenase of heart muscle. It is however not impossible that the favourable effect of cyanide may be partly due to a direct activating action on the enzyme system, such as is known to take place with certain hydrolytic systems.

Results given in Table V show that *dl*-lactate, *l*-malate, *dl*-glycerate and α-glycerophosphate reduce ferricyanide in the presence of a dialysed muscle extract to which cozymase has been added, and indicate therefore the existence of the corresponding dehydrogenases in the extract. The rate of CO_2 formation in a muscle extract-cyanide-lactate-cozymase system is shown in Fig. 1. Cozymase, which has been heated in alkaline solution, loses its catalytic activity (see Exp. 9, Table V).

The mechanism of reduction of ferricyanide is clear from facts already known. It was shown by Andersson [1934] and by Adler & Michaelis [1936] that cozymase is necessary for the activities of lactic and malic acid dehydrogenases of heart muscle. Green & Brosteaux [1936] confirmed the fact that a coenzyme forms part of the lactic dehydrogenase system, and Green [1936] confirmed the fact that coenzyme I (cozymase) forms part of the malic dehydrogenase system.

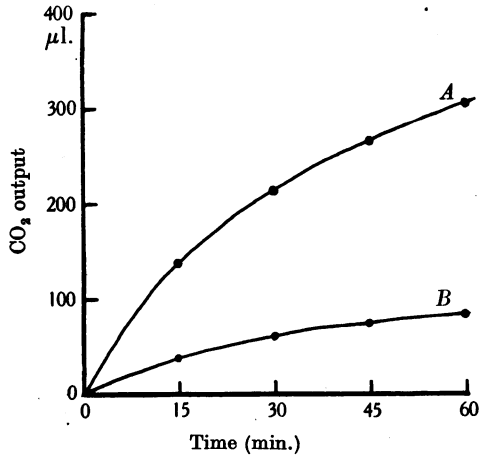

Fig. 1. *A*, 1 ml. dialysed muscle extract + NaCN (0·067 *M*) + sodium lactate (0·13 *M*) + cozymase solution (0·2 ml.) + ferricyanide. *B*, 1 ml. dialysed muscle extract + NaCN (0·067 *M*) + sodium lactate (0·13 *M*) + ferricyanide.

Adler *et al.* [1936] concluded that the coenzyme of lactic dehydrogenase is identical with cozymase, which was shown spectrophotometrically to be reduced to dihydrocozymase by the lactic acid system. The latter fact was confirmed by Green & Dewan [1937].

Since it is known [Haas, 1937] that ferricyanide oxidizes reduced cozymase in the absence of an enzyme system, it follows that the mechanism of oxidation of lactate by ferricyanide in presence of lactic dehydrogenase takes place through the intermediate formation of reduced cozymase, thus:

$$\text{Lactate} + \underset{\text{(cozymase)}}{Co\text{I}} \xrightleftharpoons[\text{dehydrogenase}]{\text{Lactic}} \underset{\text{(dihydrocozymase)}}{H_2Co\text{I}} + \text{Pyruvate}$$

$$H_2Co\text{I} + 2\text{Fe(CN)}_6''' \rightarrow Co\text{I} + 2\text{Fe(CN)}_6'''' + 2\text{H}^+.$$

In the case of malate oxidation the possibility that fumarate might be an intermediary (through the action of fumarase present in tissue extracts) is made unlikely by the experiments of Quastel & Wheatley [1931] who showed that fumarate oxidation can be accounted for on the basis that malic acid is the necessary intermediate step. This fact has been confirmed by Green [1936] and by Laki [1937]. *l*-Malate, like lactate, reduces cozymase [Euler *et al.* 1937, 2; Green & Dewan, 1937] in presence of its dehydrogenase, and Euler *et al.* [1937, 1] claim that the same phenomenon takes place with α-glycerophosphate and its dehydrogenase. That such reductions take place in a "mutase" system is clear from the observations of Green *et al.* [1937]. It follows that the oxidation of

l-malate, α-glycerophosphate and probably *dl*-glycerate by ferricyanide takes place, as with lactate, through the intermediate formation of reduced cozymase. Now it is known that pyruvate and oxaloacetate can oxidize reduced cozymase in presence of lactic and malic dehydrogenases respectively [Euler *et al.* 1936; 1937, 1; Green & Dewan, 1937]. It follows, therefore, that these α-ketonic acids can take the place of ferricyanide as oxidants of α-glycerophosphate in presence of an extract of skeletal muscle. Hence, it appears that there is no necessity to postulate a mutase acting between α-glycerophosphate and pyruvate, or between α-glycerophosphate and oxaloacetate, the conditions for such interactions being fulfilled by the demonstration of the existence of α-glycerophosphate, lactate and malate dehydrogenases in the muscle extract. These conclusions are in agreement with those of Adler *et al.* [1938].

It may also be shown that *dl*-glyceraldehyde and hexosediphosphate rapidly reduce ferricyanide in the presence of a dialysed muscle extract. The addition of cozymase is not necessary to demonstrate this reduction, although such addition increases the rate of reduction considerably with glyceraldehyde. It is possible that there is sufficient cozymase left in the extract, even after prolonged dialysis, to accomplish the catalytic reduction of ferricyanide. These results are shown in Table V, Exps. 6, 7 and 8. Glyceraldehyde is known to reduce cozymase in presence of muscle extract [Green *et al.* 1937] and the argument applying to α-glycerophosphate will apply also to glyceraldehyde.

So far as hexosediphosphate is concerned the position is not quite so clear, since reduction of cozymase has not been observed either with triosephosphate [Euler *et al.* 1937, 1] or by hexosediphosphate [Green *et al.* 1937] in the presence of muscle extracts. This failure is attributed to the fact that reduced cozymase is rapidly oxidized by glyceraldehydephosphate. There seems at present no reason to believe that the reducing action of hexosediphosphate on pyruvic acid [Braunstein & Vyshepan, 1937; Euler *et al.* 1937, 1; Green *et al.* 1937] in presence of muscle extracts is not to be attributed to the same mechanism by which α-glycerophosphate or glyceraldehyde accomplishes its reduction, i.e. through the intermediate reduction and oxidation of cozymase.

The facts given in this paper concerning the reduction of ferricyanide serve, it seems to us, to remove any doubt that dehydrogenases exist in a dialysed muscle extract and that their interaction gives rise to the properties ascribed by Green *et al.* [1937] to special mutases.

The circumstance that these dehydrogenases cannot accomplish the reduction of methylene blue makes it clear that the methylene blue-reducing systems contain a carrier transferring hydrogen from reduced cozymase to methylene blue. Evidence for such a carrier has now been obtained by Euler & Hellström [1938] and by Dewan & Green [1938].

Summary

1. A preliminary account is given of the utility of ferricyanide as a reagent for the manometric study of dehydrogenase systems.

2. Succinate and choline rapidly reduce ferricyanide in presence of their respective dehydrogenases in intact tissue slices.

3. The action of cozymase in catalysing the reduction of ferricyanide by *dl*-lactate, *l*-malate, *dl*-glycerate and *l*(+)-glutamate in presence of their dehydrogenases may be shown to take place with intact tissue slices.

4. Lysed blood cells (human, ox) form good sources of lactic and malic acid dehydrogenases, cozymase being necessary for their activities.

5. A dialysed, water-clear, aqueous extract of rabbit skeletal muscle forms an excellent source of dehydrogenases of *dl*-lactic, *l*-malic, *dl*-glyceric and α-glycerophosphoric acids. The addition of cozymase is necessary to demonstrate these activities. *dl*-Glyceraldehyde and hexosediphosphate also rapidly reduce ferricyanide in the presence of a dialysed extract of rabbit skeletal muscle. The demonstration of these dehydrogenases in a muscle extract, by the ferricyanide technique, makes it unnecessary to postulate the existence of special mutases catalysing the reduction of pyruvic or oxaloacetic acid.

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