XVIII. THE REDUCTION OF NITRATES IN ANIMAL TISSUES.

BY FREDERICK BERNHEIM
AND MALCOLM DIXON (*1851 Exhibition Senior Student*).

From the Biochemical Laboratory, Cambridge.

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It has long been known that many animal tissues contain systems capable of reducing nitrates to nitrites. Stepanow [1902] investigated the presence of nitrite in the tissues of rabbits and dogs. By means of the Griess-Ilosvay reagent he found nitrite in the white matter of the brain, the lungs, bronchi, parotids, small intestine, medullary substance of the kidney, adrenals, testicles, and lymph glands. He found it absent from the grey matter of the brain, the liver, stomach, spleen, cortex of the kidney, muscle and blood. He showed, moreover, that certain tissues which normally contained no nitrite were able to form it when ground with nitrates; and also that if the animal had been fed on food free from nitrates no nitrite was found in any of the tissues.

More recently Lipschitz [1921] has shown that the dehydrases of frog's muscle (studied by Thunberg [1920] with the methylene blue technique) were able to reduce aromatic nitro-compounds, *e.g.* *m*-dinitrobenzene, and that the nitro-compound was simply equivalent to methylene blue as a hydrogen acceptor in these systems. Bach [1911] found that nitrate could replace methylene blue in the aldehyde oxidase system, which rapidly reduced it to nitrite; and Dixon and Thurlow [1924] showed that the same was true of the xanthine oxidase system.

In view of these facts it was thought interesting to investigate the reduction of nitrates by animal tissues, in order to determine which of the known systems could use nitrate as a hydrogen acceptor.

Muscle tissue was first investigated. Preliminary experiments showed that the ability of muscle to reduce added nitrates is by no means general, and seems to be confined to a comparatively small number of species. We have found that the muscles of ox, sheep, pig, rabbit, dog and chicken are quite unable to reduce nitrates, and positive results were only obtained with rat and guinea-pig muscle.

We found, on the other hand, that the livers of all these animals reduce nitrates very readily, and the liver appears to be the main seat of the phenomenon. Rat's liver reduces five to ten times as much nitrate in a given time per unit weight as rat's muscle.

The results of these preliminary experiments led us to choose rat's muscle and ox liver as convenient tissues for the investigation of the nature of the systems responsible for nitrate-reduction, and the results obtained with these two tissues are given in the two following sections of this paper.

THE REDUCTION OF NITRATE BY RAT'S MUSCLE.

The first problem was to see whether any of the known dehydrase systems present in muscle could be responsible for the reduction. Experiments were carried out anaerobically in Thunberg vacuum-tubes as follows. Muscle from the hind legs of a freshly killed rat was finely minced and washed by shaking four times successively with 100 cc. distilled water. The washed muscle was squeezed as dry as possible, and portions of 0.3 g. were weighed out into the Thunberg tubes. 2 cc. phosphate buffer (p_H 7.5) were added to each tube, together with solutions of the appropriate hydrogen donator and acceptor. The hydrogen donators were in all cases made up in 1 % solution in water and carefully neutralised, and 1 cc. of the solution was used. With methylene blue as the hydrogen acceptor the presence in the washed muscle of systems which could oxidise the following substances was readily demonstrated: succinic, formic, citric, acetic, lactic, fumaric, maleic, malic, and glutamic acids. It was found that the washed muscle by itself reduced methylene blue slowly, even after very thorough washing, so that in all cases simultaneous experiments with and without the hydrogen donator were carried out and the rates of reduction compared. The rate of the spontaneous reduction was however, in general, small in comparison with that induced by the hydrogen donator.

The experiments with nitrate were carried out anaerobically in a similar way. 1 cc. of 5 % sodium nitrate (a considerable excess) was added to each tube instead of the methylene blue, the tubes were incubated for 2 hours at 37°, and the nitrite formed during this period was estimated roughly colorimetrically after the addition of 2 cc. of Griess-Ilosvay reagent. As before, the tubes were in all cases compared with control tubes to which no hydrogen donator had been added.

The results of these experiments showed quite definitely that the reduction of nitrate to nitrite, which still occurred with the washed muscle, could not be referred to the action of any of the dehydrase systems mentioned above, since these were all totally unable to reduce nitrate. The amount of nitrite produced by the muscle itself was not increased in the slightest by the addition of any of the hydrogen donators mentioned, and the depth of colour given by the Griess-Ilosvay reagent was always exactly the same as in the control tubes with no added donator. We had previously observed that a solution of succinoxidase (prepared from muscle by Ohlsson's [1921] method) was unable to reduce nitrates in presence of succinate. In addition to the substances mentioned, the following possible hydrogen donators were tried with the washed muscle, also with negative results: acetaldehyde, pyruvic acid,

glucose, acetone, diethyl ketone, creatinine, lecithin, cholesterol, ammonia, and various extracts of liver. The latter were prepared by extracting liver with 2 % sodium carbonate and destroying their own power of reducing nitrate by acidification and subsequent reneutralisation or by boiling (see below) and were added to the muscle in order to see whether any of the thermo- or acid-stable hydrogen donors present in the liver would increase the nitrate-reduction. Boiled and acidified muscle broths were also added to the active muscle, but no greater reduction occurred.

Gscheidlen [1874] stated that muscle which had been exercised reduced more nitrate than unexercised muscle, but we have not found this. The gastrocnemii of the rat were dissected out, and one was placed in Ringer-Locke solution and made to contract by stimulation with induction shocks until too fatigued to respond. The exercised and the control muscles were then chopped finely, a portion of each was thoroughly washed with distilled water, and 0.3 g. of each portion incubated with 2 cc. buffer + 1 cc. 5 % sodium nitrate in Thunberg tubes as before. The results were as follows:

					Nitrite reaction
Exercised muscle	...	...	...	...	++
Washed exercised muscle	...	...	...	...	++
Unexercised muscle	...	...	...	...	++
Washed unexercised muscle	...	...	...	...	++

It was impossible to detect the slightest difference in the strength of the nitrite reaction between any of the tubes, so that it appears that the nitrate-reducing system has no direct connection with the products of muscular activity.

The significance of the results described above is discussed later.

Properties of the nitrate-reducing system of rat's muscle.

The system is not readily washed out with water: after 20 washings there is considerable activity left. Nor does previous treatment with ether to break up the cell-membranes cause it to come more readily into solution.

The system can however be extracted from muscle by *M*/15 disodium hydrogen phosphate solution. Rat's muscle was chopped, washed with water, ground with the alkaline phosphate solution, and filtered. The cloudy filtrate was precipitated by saturation with ammonium sulphate and again filtered. The precipitate contained the system unimpaired, and the filtrate, when added to muscle, did not increase the reduction.

Treating the muscle with acetone does not affect the reduction. Alcohol treatment decreases it. Standing at room temperature in the air gradually destroys the reducing power, probably because the system becomes oxidised.

If the incubation with nitrate is not carried out anaerobically, but in open tubes, the amount of nitrite formed is greatly diminished. This may be due to the system using oxygen in preference to nitrate, so that the two hydrogen acceptors compete; or it may be due to a coupled oxidation of the nitrite formed in the sense of Thurlow [1925].

The system is very sensitive to acid. If brought to p_H 5 and back to neutrality the reduction is completely abolished. It is, however, more stable to alkali, and can be brought to p_H 10 and back without affecting the reduction. The actual reduction process, however, is limited to a very narrow range of p_H , and occurs only between p_H 6.5 and p_H 8, with a maximum velocity at about 7.4.

The system is destroyed by heating to 70° , but survives exposure to 60° for 5 minutes.

These properties make it extremely probable that the system is enzymic in nature, but so far we have not been able to obtain separately catalyst and substrate.

Whether the reduction goes further than the nitrite stage has not been determined.

The system is specific for inorganic nitrates, and *m*-dinitrobenzene is not reduced. This gives us the apparently paradoxical result that while this system can reduce nitrate but not nitrobenzene, the muscle dehydrases can reduce nitrobenzene but not nitrate.

Particularly interesting is the effect of cyanide on the system. The presence of $M/500$ KCN completely prevents the reduction of nitrate by the muscle, so that even after several hours no nitrite can be detected by the very sensitive Griess-Ilosvay reagent. The system is therefore cyanide-sensitive in Warburg's sense in the same way as those systems which have been shown to depend on the action of small amounts of catalytic metals. We have no data to show whether this is the case in this system or not; but it is clear that the fact of a tissue respiration process being cyanide-sensitive is not in itself sufficient reason for assuming that the effect of the cyanide is due to the paralysis of an oxygen activator, as is often done, since the cyanide may inhibit reactions in which molecular oxygen is not concerned, as here.

THE REDUCTION OF NITRATE BY LIVER.

As in the case of muscle, it was found that washing with water did not remove the nitrate-reducing power of the tissue. Washed liver was therefore used in most of the experiments. The following procedure was found to give the most satisfactory results. Half a pound of ox liver was finely minced and shaken in a flask with an equal volume of ether in order to break down the cell-membranes and render the water-washing more effective. It was then placed in a two-litre flask and washed 15 to 20 times with water, until no more haemoglobin was washed out. During this process about half the liver was washed away. The final preparation consists of small light-brown granular masses. It can be kept indefinitely in the ice chest, reduces nitrate readily, and is very convenient to use.

On investigation it was found that the reduction of nitrate by the liver was due to two distinct factors: (a) a system apparently identical with the muscle system described above, and (b) the aldehyde (and xanthine) oxidase

of liver, which is probably identical with that of milk. This latter enzyme was shown to be capable of reducing nitrate (in the presence of its substrate) by Bach [1911] and by Dixon and Thurlow [1924]. The xanthine oxidase is absent from muscle [Morgan, 1926].

It is possible to separate the two factors. On extracting the washed liver by grinding with 2 % sodium carbonate solution, filtering through muslin, and neutralising the liquid, it is found that both the factors have passed into solution. In order to obtain the xanthine oxidase free from the other factor charcoal is added, the suspension shaken for a few moments, and the charcoal filtered off with suction. A clear light-brown solution of the oxidase is obtained. The separation depends upon the fact that the nitrate-reducing factor (*a*) is readily adsorbed by charcoal, whereas the oxidase is not [Dixon and Kodama, 1926]. To obtain the former, the sodium carbonate extract is brought to p_H 10, and on reneutralising it is found that the oxidase has been completely destroyed, leaving the other factor intact.

The distinction between the nitrate-reducing system and the oxidase can also be shown by means of cyanide. The nitrate-reduction by the washed liver alone is completely inhibited, while in the presence of aldehyde that part of the reduction which is due to the oxidase is unaffected. The following is a typical experiment.

Contents of tube							Nitrite reaction
0.3 g. washed liver	+	2 cc. buffer	+	1 cc. $NaNO_3$	...	...	++
"	+	"	+	"	+ 1 cc. 1 % aldehyde	...	+++
"	+	"	+	"	+ 0.5 cc. $M/30$ KCN	...	-
"	+	"	+	"	+	+ 1 cc. aldehyde	++

On adding to the liver the possible hydrogen donors previously tested with muscle it was found that all gave entirely negative results with nitrate, with the exception of aldehyde and pyruvic acid. These two substances, and also hypoxanthine, reduced nitrate freely. The increase of reduction in presence of aldehyde and hypoxanthine is clearly due to the action of the oxidase, and was to be expected if the oxidase is the same as that in milk. Pyruvic acid, however, is quite unable to reduce nitrate, either with the oxidase alone or with the nitrate-reducing system alone; it only reduces with the liver. This reaction is in all probability to be attributed to the presence of a decarboxylase in the liver. This would convert the pyruvic acid into acetaldehyde, which would then act as a substrate for the oxidase, and so bring about the reduction. Gottschalk [1924] obtained acetaldehyde on incubating pyruvic acid with liver.

The addition of boiled or acid-treated liver broth to the active preparation of liver produced, as in the case of muscle, no increase in the nitrate-reduction.

The other properties of the nitrate-reducing system of liver are similar to those of the system in muscle. Treatment with acetone does not affect the reduction, but alcohol partially destroys it. It is thermolabile, and is destroyed by heating to 70° , but not by heating to 60° for 5 minutes. It is

acid-sensitive, and is completely destroyed at p_H 5; but, on the alkaline side, p_H 10 leaves it quite unaffected. It is cyanide-sensitive, as mentioned above. The similarity in properties strongly suggests that the nitrate-reducing systems in muscle and liver are identical.

Attempts have also been made to separate catalyst and substrate in the liver system by precipitation and other methods, but without success.

The washed liver contains very little glutathione, to judge from the nitroprusside reaction, but it will very readily reduce oxidised glutathione. A preparation which gives an almost negative nitroprusside test will, after having been incubated anaerobically for a short period with oxidised glutathione, give a very strong reaction. This fact may be of importance in the study of the glutathione system. This reduction is unaffected by boiling the liver, whereas of course the nitrate-reduction is completely destroyed, so that the two reductions cannot be due to the same factors.

The thoroughly washed liver is still able to reduce methylene blue, but we are as yet unable to say whether part or all of this reducing power is due to the nitrate-reducing system or not. The liver also reduces the series of dyes used by Mansfield Clark as r_H indicators. The results obtained with these dyes, however, are somewhat unexpected and difficult to explain, though perfectly definite. They are as follows. The dyes were freshly made up in aqueous solution in concentrations equivalent to the 1/5000 methylene blue solution used. (The names and r_H ranges of the dyes have been previously given in this *Journal* [Dixon, 1926, p. 714], and they are here referred to simply by their series-numbers. They are numbered in ascending r_H order, so that No. 9 is theoretically the easiest and No. 1 the most difficult to reduce. No. 3 *a* is methylene blue.) The reductions were carried out in Thunberg tubes as before, and 0.3 g. of thoroughly washed liver + 2 cc. buffer + 1 cc. of the dye were used in each tube. The table gives the reduction-times (in minutes) with and without the addition of 1 cc. 1 % acetaldehyde.

Dye No.	Reduction-time		Dye No.	Reduction-time	
	Alone	+ aldehyde		Alone	+ aldehyde
1	120	70	5	45	50
2	420	100	6	17	35
3	480	100	7	20	38
3 <i>a</i>	60	40	8	25	54
4	45	50	9	8	25

It will be seen that while the first four dyes behave normally in being reduced more rapidly in the presence of aldehyde than in its absence, reduction of the last six dyes is actually *slowed* by the aldehyde. The cause of this is not clear, but it is possible that there is some active reducing system in the liver whose potential is such that it can reduce dyes 4 to 9, but cannot reduce dyes 1 to 3 *a*. If now this system is inhibited by the presence of aldehyde, results similar to those found will be produced. For, in the case of the last six dyes, where the reduction may be mainly due to this system, the inhibition produced by the action of the aldehyde on the system may

obscure the acceleration produced by the action of the aldehyde oxidase. With the first four dyes, on the other hand, the system plays no part, and consequently the action of the oxidase shows up. This is merely a suggestion, but it is not easy at the moment to see any other explanation.

DISCUSSION.

Though the significance of some of the facts given in this paper is not yet clear, a number of interesting points are brought out by the work.

It appears fairly certain that the nitrate-reducing system in muscle, and the corresponding system in liver, cannot be identical with any of the hitherto described reducing enzymes, and it seems that we must attribute the reduction to a new enzyme system. As to its significance in connection with tissue respiration processes we can of course say little as yet.

As we have pointed out above, the system is very active in the livers of all the animal species examined; but the fact that it is absent from the muscles of all but two of the species affords another example of the curious and apparently haphazard distribution of some of the systems concerned in biological oxidations (compare the xanthine oxidase [Morgan, 1926]).

The fact that the Thunberg tissue dehydrases are unable to reduce nitrate is a fact of considerable importance in considering the ability of nitrate to act as a direct hydrogen acceptor. Dixon and Thurlow, working on the milk oxidase, believed that nitrate was simply equivalent to methylene blue as a hydrogen acceptor, and it is possible that this is the case in the system with which they were dealing. It is clear, however, that the two substances are by no means always equivalent.

We have not sufficient data as yet to decide whether the failure of nitrate to replace methylene blue in the Thunberg dehydrase systems is due to an insufficiently high reducing power in these enzyme systems, to a difference in the accessibility of the two substances to the active enzyme surface, or to the lack of a nitrate-activating catalyst of the type studied by Quastel, Stephenson and Whetham [1925]. Each of these possibilities would seem to provide an adequate explanation.

The first possibility involves the assumption that methylene blue is more easily reduced than nitrate, and that the dehydrases, with the exception of the aldehyde and xanthine oxidase, have reducing powers greater than that necessary to reduce methylene blue but less than that necessary to reduce nitrate. The xanthine oxidase seems to be a particularly strongly reducing system compared with the other dehydrases. Its reducing power must be much higher than that of the succinoxidase for instance, for while the latter system comes to an equilibrium with methylene blue considerably before the dye is completely reduced [Thunberg, 1925], the xanthine oxidase reduces methylene blue completely (*i.e.* more than 99.9999 %), as shown by Clark [1927]. Although a number of systems are known which will reduce methylene

blue but not nitrate, we are not aware of any¹ which will reduce nitrate but not methylene blue.

The possibility that the difference in behaviour is due to a difference in accessibility must be borne in mind; but, although it may explain the facts in the systems under consideration, it cannot account for the difference in behaviour with homogeneous systems (*e.g.* cysteine, referred to below).

In connection with the third possibility it should be pointed out that, while it seems probable that the muscle dehydrases may be able to reduce nitrate only when the latter is activated by a catalyst, it seems quite possible nevertheless that nitrate may be directly reduced without activation in such systems as the xanthine oxidase. Quastel, Stephenson and Whetham used as a test of nitrate-activation the ability of the activated nitrate to oxidise reduced methylene blue, which nitrate by itself is unable to do. We have found that using this test no nitrate-activation can be detected in the xanthine oxidase system: in one limb of a branched vacuum-tube was placed sodium nitrate solution, and in the other limb xanthine oxidase solution in phosphate buffer together with methylene blue and an amount of hypoxanthine just sufficient to reduce the latter; after careful evacuation of the tube and allowing to stand until the methylene blue was reduced, the nitrate solution was immediately run into this solution by tilting the tube. Not the slightest trace of blue colour appeared. If then the oxidation of leuco-methylene blue is a reliable indication of the presence of activated nitrate it would seem that the xanthine oxidase system does not contain a nitrate-activating catalyst and that the oxidase is able to reduce nitrate directly. If the nitrate is added initially with the methylene blue the reduction of the latter is slowed, presumably because the nitrate competes with the methylene blue as a hydrogen acceptor.

If it is assumed that nitrate invariably requires activation before it can be reduced in the tissues it is clear that the catalyst responsible must be associated in a very special way with particular oxidase systems in the cell. For example, the succinic oxidase in, say, a liver extract cannot reduce nitrate, while *in the same solution* the aldehyde oxidase reduces nitrate freely, so that the activator, if present, must be in intimate association with the latter system in such a way that its action is not available for other systems. The same applies also to the nitrate-reducing system in muscle.

Nitrates differ from methylene blue in giving no oxidation-potential at a platinum electrode, in this respect resembling the aromatic nitro-compounds. The latter are nevertheless easily reduced, both *in vitro*, for example by —SH compounds (cysteine, glutathione, etc.), and also by the dehydrases. Nitrates are not reduced by cysteine, unless a nitrate-activating catalyst is present (Quastel, Stephenson and Whetham). They are, however, reduced *in vitro* by such reducing systems as zinc dust suspended in *neutral* solution—a system which has reducing properties which very closely parallel those of the xanthine

¹ One of us (F. B.) will deal with the aldehyde oxidase of the potato in a paper to be published shortly.

oxidase system. For instance, in addition to the reduction of the usual dyes, etc. they both take up molecular oxygen, forming hydrogen peroxide, which is subsequently reduced to water; the oxygen uptake is not prevented by cyanide; they both reduce nitrates and aromatic nitro-compounds; and they both fail to reduce the —SS— group¹. Other points of similarity might be mentioned. The Thunberg dehydrases, on the other hand, would seem rather to correspond in reducing properties with —SH compounds. They fail to reduce nitrates in the absence of a special catalyst; the oxidation of the —SH group by molecular oxygen is abolished by small amounts of cyanide [Warburg and Sakuma, 1923; Harrison, 1924], which is true also of, at any rate, the oxidation of succinic acid by the succinoxidase [Fleisch, 1924]; and both —SH compounds and the succinoxidase system reduce oxygen some hundreds of times faster than methylene blue, which is not the case with the xanthine oxidase.

We would suggest provisionally that, while the majority of the dehydrases can reduce nitrate only in the presence of a special catalyst, certain of them are able to reduce nitrate directly in the absence of such a catalyst.

SUMMARY.

1. While the livers of all the animals studied reduce nitrate freely, nitrate-reduction in muscle is confined to the rat and the guinea-pig. A study of nitrate-reduction in rat's muscle and ox liver has been made.

2. The reduction of nitrate by muscle cannot be referred to any of the known reducing systems present. The dehydrases of muscle are incapable of reducing nitrate. No hydrogen donator has been found which will increase the reduction of nitrate by washed muscle.

3. The properties of the nitrate-reducing system are described. It appears to be enzymic in nature.

4. The nitrate-reduction is completely abolished by the addition of $M/500$ cyanide.

5. In liver the nitrate-reduction is due to two factors: (a) a system having the same properties as that in muscle, and (b) the aldehyde (xanthine) oxidase, probably identical with that in milk. These two factors can be separated. No hydrogen donator, other than the substrates of the oxidase, has been found which will increase the reduction of nitrate by liver.

6. These facts are discussed in relation to theories of biological oxidation processes.

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¹ Zinc dust in acid solution of course reduces the —SS— group.

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