CXXXVIII. HEPATIC OXIDATION OF CHOLINE AND ARSENOCHOLINE

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It was shown by Mann & Quastel [1937] that choline is oxidized by slices or extracts of rat liver and kidney. The oxidation product was isolated and identified as betaine aldehyde. Some evidence was obtained that betaine aldehyde is also oxidized by the same tissues though at a much slower rate than choline and it was suggested that the oxidation product in this case is betaine. Bernheim & Bernheim [1933] observed an increase in oxygen uptake when acetylcholine was added to extracts of liver and kidney of rat and cat and suggested that it was due to choline oxidation. The same authors in 1938 [cf. also Bernheim & Webster, 1937] arrived at the conclusion that betaine aldehyde is formed, without, however, isolating it. They showed that synthetic betaine aldehyde is oxidized by liver extract, the rate of oxidation being dependent upon *pH*. At *pH* 7·8 it is oxidized presumably to betaine but at *pH* 6·7 the aldehyde suffers little change.

In the present work it is shown that one of the components of the choline-oxidizing system is a typical dehydrogenase which can bring about the oxidation of choline anaerobically, provided that a suitable hydrogen acceptor is present, and which is insensitive to low concentrations of cyanide though the complete system is cyanide-sensitive. Some evidence is also put forward which indicates that the other components of the oxidizing system are cytochrome and indophenol oxidase. The oxidation of arsenocholine by the same system is also described.

Methods

Choline and arsenocholine¹ were both used as the chlorides. The enzyme preparations employed were made from rat liver by the method already described by Mann & Quastel [1937]. For the anaerobic experiments with ferricyanide which were carried out in bicarbonate buffer a dialysed preparation made according to the description of Bernheim & Bernheim [1938] was employed. All manometric experiments were carried out in the Warburg apparatus at 37°, the total volume of medium in each vessel being always 3·0 ml. Aerobic experiments were carried out in a sodium phosphate buffer medium of final concentration 0·06 *M* at *pH* 7·4 in air. Anaerobic experiments were carried out in 0·025 *M* bicarbonate in an atmosphere of 95 % N₂ + 5 % CO₂. Sodium ferricyanide was used in these experiments as the hydrogen acceptor according to Quastel & Wheatley [1938], and the oxidation was followed by measuring the CO₂ output.

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The oxidation of arsenocholine

The effect of addition of arsenocholine on the O_2 uptake of rat liver extract is shown by the curve of Fig. 1. The required amounts of arsenocholine chloride solution were placed in the side tubes of the Warburg vessels and tipped into the main compartment immediately after the initial readings had been taken. In the figure the values of the O_2 uptakes in the first 15 min. are plotted against the amount of arsenocholine chloride present. It will be observed that the optimum concentration of substrate is not reached even with 40 mg. arsenocholine chloride in a total volume of 3 ml. (i.e. $0.07 M$). With choline chloride, as has previously been shown, the optimum concentration is of the order of 1 mg. per ml. (i.e. $0.007 M$). Hence the enzyme has a comparatively low affinity for arsenocholine.

Fig. 1.

Fig. 2.

Fig. 1. The effect of addition of arsenocholine on the O_2 uptake of rat liver extract.

Fig. 2. The effect of 5 mg. choline chloride, 5 mg. arsenocholine chloride and a mixture of 5 mg. arsenocholine chloride + 5 mg. choline chloride on the O_2 uptake of the liver extract.

That the dehydrogenase concerned in the oxidation of arsenocholine is identical with choline dehydrogenase is indicated by the fact that competition occurs between choline and arsenocholine as substrates. This is shown by the curves of Fig. 2 where the O_2 uptakes produced by the addition of 5 mg. choline chloride, 5 mg. arsenocholine chloride and by a mixture of 5 mg. arsenocholine chloride + 5 mg. choline chloride to the liver extract are plotted. Under these initial conditions the additional O_2 uptake produced by choline is rather more than double that produced by arsenocholine. The initial concentration of choline is sufficient to saturate the enzyme. When choline and arsenocholine are both present the O_2 uptake is not additive as would be expected if the substrates were activated by different enzymes but on the contrary is slightly less than when choline alone is present. The diminution is small owing to the much greater affinity of the enzyme for choline than for arsenocholine. As oxidation proceeds the residual choline becomes insufficient to saturate the enzyme and hence the

uptake becomes higher where both choline and arsenocholine are present together.

Judging from the total O_2 uptake the oxidation of arsenocholine follows a course analogous to that of choline. The total extra O_2 uptake per mg. arsenocholine chloride added is in the neighbourhood of the 112 μ l. necessary if 1 mol. of arsenocholine requires 1 mol. of O_2 . This would suggest that the final product of oxidation is arsenobetaine. Thus in the experiment illustrated by the curves of Fig. 3 the value is 107 μ l. Furthermore in the early stages of the oxidation a substance is formed which reacts with 2:4-dinitrophenylhydrazine. This may be conveniently demonstrated by adding to 1 ml. of the reaction mixture 1 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in *N* HCl, keeping the mixture at room temperature for 30 min. and making alkaline by addition of 2.5 ml. *N* NaOH when a deep red colour is produced indicating the presence of a hydrazone. The substance reacting with the phenylhydrazine is presumably a

Fig. 3. The effect of semicarbazide on the oxidation of choline and arsenocholine.

arsenobetaine aldehyde. During the later stages of the oxidation the reaction with 2:4-dinitrophenylhydrazine diminishes in intensity and finally becomes no greater than that of the control without added arsenocholine. It appears therefore that, as with choline, a comparatively rapid oxidation to the aldehyde occurs, the latter being oxidized at a much slower rate to the corresponding acid. It is evident however that in the case of arsenocholine a secondary reaction takes place. During the oxidation a strong garlic odour suggestive of trimethylarsine is produced, and acid permanganate placed in the side tubes of the Warburg vessels is decoloured, indicating the formation of a volatile reducing substance.

Use of semicarbazide as fixative

The oxidations both of choline and of arsenocholine may be arrested at the aldehyde stage if semicarbazide is used as a fixative. This is shown by the curves of Fig. 3 which illustrate the course of oxidation of choline and arsenocholine in presence and absence of semicarbazide. The concentration of semicarbazide used was 0.1 *M* in the choline experiment and 0.05 *M* in the arsenocholine

experiment. The amount of substrate added in each case was 1 mg. of the chloride. The control figures for the O_2 uptake of the enzyme preparation in presence and absence of semicarbazide have been subtracted and the extra O_2 uptake due to the added substrate is plotted. The experiments with choline and arsenocholine were carried out on different days and have been fitted on to the same figure for convenience. It will be observed that in presence of semicarbazide the O_2 uptakes with both substrates correspond roughly with the theoretical value assuming that the substrate molecule takes up 1 atom of oxygen. In absence of semicarbazide however the O_2 uptake attains values nearly equivalent to 1 mol. O_2 per mol. of substrate. Thus in presence of semicarbazide the actual figures obtained are 71 and 62 μ l. for choline and arsenocholine respectively, whereas the theoretical figures are 80 and 56 μ l. In absence of semicarbazide the figures obtained are 140 and 107 μ l. and the theoretical figures 160 and 112 μ l. Dinitrophenylhydrazine tests carried out at the conclusion of the experiments in the manner already described give strongly positive results where semicarbazide has been present and weak or negative ones in its absence. This indicates that the effect of semicarbazide is due to fixation of betaine aldehyde and arsenobetaine aldehyde.

It has already been reported that during the oxidation of arsenocholine volatile reducing substances are formed, as shown by the reduction of acid permanganate placed in the side tubes of the manometric vessels. If the oxidation is carried out in presence of semicarbazide this reduction of acid permanganate does not occur. Either the secondary reaction does not take place in presence of semicarbazide or the products of this reaction also are bound by semicarbazide. Attempts to isolate arsenobetaine aldehyde from the reaction mixture have so far only yielded mixtures and it is evident that a secondary decomposition takes place. In the case of choline oxidation however where apparently no secondary reaction occurs it should be possible to simplify the method used by Mann & Quastel [1937] for separation of betaine aldehyde either by the use of semicarbazide as a fixative or by utilizing the finding of Bernheim & Bernheim [1938] that at pH 6.7 the oxidation of betaine aldehyde proceeds only very slowly.

Reduction of ferricyanide

The properties of choline dehydrogenase may be conveniently studied by the use of ferricyanide as a hydrogen acceptor as shown by Quastel & Wheatley [1938]. The experiments are carried out in a bicarbonate (0.025 *M*) medium in an atmosphere of 95 % N_2 + 5 % CO_2 , and the reaction is followed by measuring the CO_2 displaced from the bicarbonate by the acid produced by reduction of the ferricyanide according to the equations

A typical curve illustrating the oxidation of choline by this method is given in Fig. 4. The amount of choline chloride used in this experiment was 0.5 mg. A control vessel containing 0.5 mg. choline chloride and ferricyanide but no enzyme preparation showed a CO_2 output of only 9.4 μ l. during the experimental period.

Bernheim & Bernheim [1938] report that cyanide in fairly large concentrations inhibits the oxidation of choline but that the inhibition is of rather short duration. This is found not to be the case when the inner cups of the manometer vessels contain the appropriate potash-cyanide mixture to prevent

change in the concentration of cyanide in the reaction mixture during the course of the experiment [Mann & Quastel, 1937]. In anaerobic experiments however using ferricyanide as the hydrogen acceptor concentrations of cyanide which produce almost complete inhibition of choline oxidation aerobically here show little or no inhibition. The effects of cyanide aerobically and anaerobically

Fig. 4. The anaerobic oxidation of choline with ferricyanide as a hydrogen acceptor.

Fig. 5.

Fig. 5. The complete inhibition of aerobic oxidation of choline and arsenocholine by $10^{-3} M$ NaCN.

Fig. 6.

Fig. 6. The effect of $10^{-3} M$ NaCN on the anaerobic oxidation of choline and arsenocholine.

on choline and arsenocholine oxidation are illustrated by the curves of Figs. 5 and 6. Sodium cyanide was used in both aerobic and anaerobic experiments at a final concentration $10^{-3} M$. The amount of choline and arsenocholine chloride added to each vessel was 10 mg. The control figures for the CO_2 output or O_2 uptake of the enzyme preparation alone and in presence of cyanide have been

subtracted from the values obtained in presence of choline and arsenocholine. Since the inhibitory effect of cyanide is exercised aerobically but not anaerobically it is apparent that the component of the oxidation system which is cyanide-sensitive is not the choline dehydrogenase. The latter is comparatively cyanide-insensitive though results of experiments with higher concentrations of cyanide indicate some sensitivity to concentrations of the order of $10^{-1} M$.

Reduction of cytochrome c

Choline dehydrogenase in presence of either choline or arsenocholine brings about a rapid reduction of cytochrome *c*.¹ This has been established by anaerobic experiments carried out in Thunberg tubes, the reduced cytochrome being identified by its absorption spectrum by means of the microspectroscope. One such experiment may be described here.

A rat liver was chopped with scissors, washed repeatedly with tap water by decantation, and the residue was ground in a mortar with sand and 10 ml. 0.1 *M* phosphate buffer pH 7.4. The mixture was squeezed through muslin and the resulting suspension was centrifuged. The precipitate was mixed with water, again centrifuged, and finally suspended in 0.1 *M* phosphate buffer pH 7.4. An amount of the suspension equivalent to 0.1 g. liver was placed in each of three Thunberg tubes. One tube contained no added substrate, the second contained 10 mg. choline chloride and the third 10 mg. arsenocholine chloride. To each tube 0.15 ml. of a solution of cytochrome *c* (5 mg. per ml.) was added and the total volume was made up to 3 ml. in each case. On evacuation at room temperature the tubes containing choline and arsenocholine showed the spectrum of reduced cytochrome *c* at once. A strong band was seen at about 550 $m\mu$. and a weak one at about 520 $m\mu$. On shaking the suspensions with air the bands of reduced cytochrome quickly disappeared but reappeared in a few seconds when the tubes were again evacuated. Neither the enzyme preparation alone nor the heat-inactivated enzyme in presence of choline showed any reduction of cytochrome *c* under anaerobic conditions in Thunberg tubes after 15 min. at room temperature. Guinea-pig liver preparations made in a similar manner which do not oxidize choline [Mann & Quastel, 1937] showed no reduction of cytochrome *c* in presence of choline, though a rapid reduction was obtained in presence of succinate.

These results make it evident that choline dehydrogenase in presence of its substrates, choline or arsenocholine, can accomplish the reduction of cytochrome *c*, and there is every reason to believe therefore that the complete choline oxidase system consists of choline dehydrogenase, cytochrome *c* and cytochrome oxidase (indophenol oxidase). Thus the transfer of hydrogen may be expressed by the scheme:

Such a scheme accounts for the cyanide-insensitivity of the anaerobic oxidation of choline or arsenocholine by ferricyanide and the cyanide-sensitivity of the oxidation by oxygen. Whether the participation of a carrier or coenzyme is necessary between choline dehydrogenase and cytochrome is not known but there is no evidence for this so far. Choline dehydrogenase, like succinic acid

¹ We are much indebted to Prof. Keilin for a specimen of cytochrome *c*.

dehydrogenase in tissue extracts reduces ferricyanide rapidly, whereas, as shown by Quastel & Wheatley [1938], the rapid reduction of ferricyanide by lactate, *l* malate etc., under similar conditions, requires the addition of cozymase. Prolonged dialysis of the enzyme preparation has little effect on the activity and addition of cozymase after such treatment does not lead to any increase in the rate of oxidation. Attempts to separate the dehydrogenase from cytochrome and then to reconstitute the complete system by addition of cytochrome have as yet been unsuccessful. This is doubtless due to the unusually great instability of the dehydrogenase. The routine methods of preparation, such as precipitation of tissue extracts with acetone or ammonium sulphate, or by acidification, apparently all cause complete destruction of the dehydrogenase.

Some properties of choline dehydrogenase

The conclusion that choline dehydrogenase activates both arsenocholine and choline is borne out not only by the fact that competition takes place between the substrates for the enzyme but by the fact that tissue extracts which fail to oxidase choline also fail to oxidase arsenocholine. For example, a guinea-pig liver extract which took up 36.3 μ l. O_2 in 1 hr. at 37° took up 36.2 μ l. under the same conditions in presence of 20 mg. arsenocholine chloride.

Choline dehydrogenase is distinct from alcohol dehydrogenase. Liver extracts, prepared for the experiments cited in this paper, oxidize both choline and alcohol. When a mixture of these two substrates is present the O_2 uptake is additive. This is shown by the following figures:

	μ l.
O_2 uptake by rat liver extract alone in 45 min. at 37°	64.9
" " + choline (10 mg.)	283.7
" " + alcohol (5 mg.)	114.1
" " + choline (10 mg.) and alcohol (5 mg.)	332.7
The theoretical figure for additive oxygen uptakes	= 332.9

Bernheim & Bernheim [1938] showed that a dialysed preparation of choline oxidase is unable to oxidize alcohol, and conversely that an aqueous extract of acetone-dried liver oxidizes alcohol but not choline. Clearly the $—CH_2OH$ group is not a competing group for the enzyme. On the other hand, the group $>NR_3$ ($R=H$ or CH_3) seems to be of primary importance for the attachment of the substrate to the enzyme. This is shown by the fact that NH_4^+ , $NH(CH_3)_3^+$, and betaine inhibit choline oxidation. Typical curves illustrating the inhibitory activities of these substances are shown in Figs. 7, 8 and 9. It will be observed that ammonium chloride decreases the O_2 uptake of the liver extract alone. This is possibly due to inhibition of oxidation of choline. Access to the enzyme would appear to depend on the presence of the $>NR_3$ group in the substrate.

As is evident from the oxidation of arsenocholine by choline dehydrogenase, the N atom of the grouping in question may be replaced by As, with loss, however, in affinity for the enzyme. The Michaelis constant of the enzyme for choline is about 0.0012 *M* and for arsenocholine apparently about 0.008 *M*.

It is of interest that, as shown by Roepke [1937], acetylarsenocholine is hydrolysed by choline esterase and has an affinity for that enzyme as great as that of acetylcholine. Both choline and arsenocholine inhibit choline esterase, both substrates having approximately equal affinities. Thus substitution of N by As still clearly allows accessibility to the enzyme to take place.

DISCUSSION

The evidence presented in this paper shows that choline and arsenocholine are both oxidized by the same enzyme system to the respective aldehydes. The enzyme system in question consists partly of a typical dehydrogenase and partly

Fig. 7. Inhibition of choline oxidation by ammonium chloride.

Fig. 8. Inhibition of choline oxidation by trimethylamine.

Fig. 9. Inhibition of choline oxidation by betaine.

of the cytochrome-indophenol oxidase system. Cytochrome *c* undergoes reduction by choline or arsenocholine in presence of the dehydrogenase and there is as yet no evidence that a coenzyme takes part in this reduction. It appears therefore that choline dehydrogenase, together with its substrate, like succinic acid dehydrogenase, reduces cytochrome directly. It must be concluded that, in organs in which choline oxidation takes place, that part of the respiration

which includes choline combustion proceeds through the intermediate action of cytochrome. That the enzyme activating choline or arsenocholine is a typical dehydrogenase is shown conclusively by the rapid reduction of ferricyanide which takes place under anaerobic conditions and which is not cyanide-sensitive.

It is of interest that arsenocholine is oxidized by choline dehydrogenase although the substitution of N by As has obviously lowered the affinity of substrate for the enzyme. The grouping $>NR_3$ ($R=H$ or CH_3) seems to be primarily important for access of substrate to the enzyme as shown by the inhibitions effected by ammonium and trimethylammonium ions and by betaine.

SUMMARY

1. Arsenocholine, like choline, is oxidized by choline oxidase in rat liver to the corresponding aldehyde. This is followed probably by oxidation to arsenobetaine. The substitution of N by As reduces the affinity of the substrate for the enzyme.

2. Secondary decomposition of arsenocholine takes place in presence of rat liver with liberation of a volatile reducing compound having a strong garlic odour (? trimethylarsine).

3. In presence of semicarbazide, fixation of betaine aldehyde, and arsenobetaine aldehyde, takes place, kinetic studies showing an O_2 uptake corresponding to the formation of these substances.

4. Choline oxidase consists partly of a typical dehydrogenase. This is shown by the fact that rat liver extracts, in presence of choline or arsenocholine, rapidly reduce sodium ferricyanide under anaerobic conditions. This system is cyanide-insensitive and may be studied manometrically in a bicarbonate medium.

5. Choline dehydrogenase, in presence of choline or arsenocholine, rapidly reduces cytochrome *c* at room temperature. There is no evidence that a coenzyme is required for the reduction of cytochrome by choline dehydrogenase in presence of its substrates.

It is inferred that the choline oxidase system consists of choline dehydrogenase, cytochrome *c* and cytochrome (indophenol) oxidase.

6. Choline oxidation is inhibited by ammonium ions, trimethylammonium ions and by betaine. It is concluded that the group $>NR_3$ ($R=H$ or CH_3) is primarily important for the access of the substrate to the enzyme.

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