CXVI. THE OXIDATION OF CHOLINE BY RAT LIVER

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THE importance of choline, both as a dietary constituent for the prevention and cure of fatty livers, and in the form of acetylcholine for the transmission of nervous impulses suggests that its intermediary metabolism in the animal body may present features of unusual interest. While much work has been done on this subject in the past and many suggestions have been made as to the way in which choline is transformed by the animal, the evidence which has been obtained is not sufficiently conclusive to establish any of the hypotheses put forward.

The simplest suggestion that has been made is that choline is oxidized at the alcoholic group with the formation of betaine, the corresponding acid. There appears to be no experimental evidence in support of this view. It depends upon the observation that choline and betaine are frequently found together in plants, and the fact that the oxidation of choline to betaine is readily brought about by the action of oxidizing agents. However, despite the widespread occurrence of choline in the tissues of higher animals, betaine appears to have been isolated from such a source on only one occasion when Bebeschin [1911] reported the isolation of betaine from ox liver.

A view which receives more support suggests that as a preliminary to oxidation, choline is first split up into trimethylamine and glycol. Such a splitting of choline is known to be brought about by the action of certain micro-organisms. Thus Ackermann & Schutze [1910; 1911] reported the production of trimethylamine from choline by *B. prodigiosus*, and it appears possible that this change may be also brought about by animal tissues, since Toda [1934] claims that trimethylamine is produced from choline by liver pulp. According to Hoesslin [1906] the trimethylamine so produced is demethylated and converted into urea and formic acid. He found that after administration of choline to rabbits, orally or subcutaneously, the formic acid content of the urine increased, though there was no trace of unchanged choline and no increase in trimethylamine content.

From the recent work of Lintzel [1934] it appears doubtful whether, in man at any rate, splitting of choline into trimethylamine and glycol occurs. Lintzel found that when trimethylamine is fed to man, it appears quantitatively in the urine as trimethylamine oxide. When choline is fed, however, it causes little increase in trimethylammonium compounds in the urine, though a small proportion of it may appear in the urine unchanged and there may be a small increase in the trimethylamine oxide content. Lintzel concludes that choline must undergo demethylation in some such way as that suggested by Riesser [1913; 1914].

Riesser found that on injection of choline into rabbits, a significant rise in the creatine content of the muscle occurred together with an increase in urinary creatinine. He concludes that choline is a precursor of creatine and suggests that the conversion is brought about by a partial demethylation of choline followed by condensation with urea. The condensation product would undergo oxidation to creatine. Satta [1914] observed no increase in urinary creatinine in animals fed on lecithin and postulated a complete demethylation of choline leading to the formation of aminoethyl alcohol. According to Guggenheim & Löffler [1916] the latter compound is rapidly oxidized when perfused through surviving liver.

The above suggestions as to the mode of breakdown of choline in the animal organism have been derived mainly from experiments performed on intact animals by feeding or injection of choline. Experiments with tissue slices and extracts have shown that the addition of choline or acetylcholine may bring about an increase in the rate of respiration of the liver or kidney tissue of certain animals. Thus Bernheim & Bernheim [1933] investigated the increase in oxygen uptake produced by the addition of acetylcholine to extracts of liver or kidneys of rats or cats. Since the acetylcholine disappeared and the addition of acetate produced no similar increase in the rate of oxygen uptake, they concluded that the choline was oxidized. But the disappearance of acetylcholine merely indicates the presence of choline esterase, and the possibility that choline is merely acting as an activator of respiration rather than as a substrate was not excluded. Trowell [1935], working with rat liver slices and with preparations of washed liver, also came to the conclusion that choline was oxidized by this tissue, but he was mainly concerned with the effect of choline on fat metabolism and made no attempt to follow the course of choline oxidation.

In the present work, the fact that the increased oxygen uptake produced by the addition of choline to rat liver slices and extracts is due to oxidation of choline is established and the identification of the oxidation product is described.

Methods

Choline chloride was used throughout. The tissue used was rat liver either in the form of slices or as an extract prepared by a method similar to that of Bernheim & Bernheim [1933]. The tissue was ground in a mortar with sand, and $M/5$ phosphate buffer pH 7·4, varying in amount from 1 to 5 ml. per g. of tissue according to the concentration of extract required, was stirred in. After the mixture had been allowed to stand for 15 min. at room temperature, it was squeezed through muslin. The resulting suspension could be conveniently measured by pipette into the experimental flasks.

Experiments in which oxygen uptake was measured were carried out in the Warburg apparatus at a temperature of 37°. The total volume of medium in each vessel was always arranged to be 3·0 ml. When tissue slices were used, the medium had the composition: sodium phosphate buffer (pH 7·4) 0·03 M, KCl = 0·002 M, $CaCl_2$ = 0·001 M, NaCl = 0·13 M. With tissue extracts the final concentration of sodium phosphate buffer was arranged to be 0·06 M, and no other salts were added. In experiments with tissue slices the manometric vessels were filled with oxygen.

Estimation of choline

Two methods were used for the estimation of choline.

Reineckate method. This method depends upon the precipitation of choline as the reineckate which is insoluble in ice-cold water and in absolute alcohol. It was

used by Kapfhammer & Bischoff [1930] for the isolation of choline and acetylcholine from animal tissues. Beattie [1936] used the precipitation as the basis of a colorimetric method of estimation of choline which was used in the present work. The method consists in precipitating the choline by the addition of excess of ammonium reineckate solution, dissolving the washed precipitate in acetone, and estimating the choline reineckate by comparing the colour of the acetone solution in a colorimeter with that of a methyl red solution previously standardized against pure choline reineckate. Experimental solutions were freed from protein before precipitation of the reineckate by addition of an equal volume of 20% trichloroacetic acid.

The periodide method. Choline is precipitated as periodide by Florence's reagent—a solution of iodine in potassium iodide. This has been utilized as a means of estimating choline by Stanek [1906] who estimated the nitrogen of the precipitate and by Roman [1930] who estimated the iodine of the precipitate. These methods are, however, unspecific. Booth [1935] has used the reagent for a microchemical test for choline which depends on the observation of the formation of the characteristic choline periodide crystals under the microscope. He has shown that the formation of crystals occurs at extremely low concentrations of choline. This observation has been utilized in the present work as the basis of a rough but rapid estimation of choline. In principle this consists in standardizing the reagent by observing the lowest concentration of choline at which the periodide formation is detectable. The experimental solution of unknown choline concentration is then diluted until the same point is reached, when, knowing the sensitivity of the reagent, the amount of choline present may be calculated.

The reagent is prepared by adding 2·54 g. iodine to 1·65 g. potassium iodide dissolved in 30 ml. water. When first prepared it is relatively insensitive but after 24 hr. it generally gives a positive test with a solution of 1 mg. choline chloride in about 150 ml. water. Further standing produces a very slow increase in sensitivity. For standardization such a volume of choline chloride solution as contains 1 mg. choline chloride is pipetted into a 250 ml. measuring cylinder. The solution is diluted to 120 ml. and after mixing, 1 drop is put on a microscope slide and then 3 drops of Florence's reagent are added. Further tests are also made at dilutions of 130, 140 ml. etc. The test samples are then examined under the microscope for choline periodide crystals. It is found that with the more concentrated choline solutions, minute crystals may be seen all over the field, while with the more dilute solutions only a few scattered crystals are to be seen. The change over is fairly sharp. Thus, for example, the dilution of 140 ml. may give a good positive test, while the dilution of 150 ml. and subsequent dilutions give only a few scattered crystals or none at all. The reagent will then detect a concentration of choline of 1 in 140,000. In such dilutions the periodide crystals may take up to 5 min. to develop. They slowly decompose on prolonged standing at room temperature. With a choline solution of entirely unknown strength a series of tests at widely different dilutions are first carried out to obtain some idea of the amount of choline present. Then a volume which contains about 1 mg. is pipetted into the measuring cylinder and a series of tests at different dilutions are carried out as for the standardization. From the highest dilution which gives a positive test; the amount of choline present may be calculated, knowing the sensitivity of the reagent. Experimental solutions were freed from protein by the addition of an equal volume of 20% trichloroacetic acid before the estimations were carried out.

The accuracy of the method is, of course, not great. Probably the error is in some cases as high as 10%. But whereas by the reineckate method of estimation

compounds related to choline, such as acetylcholine, trimethylamine and betaine, are all estimated as choline to a greater or less extent according to the solubility of their reineckates, the periodide method just described is apparently specific for choline. Related compounds react with the periodide reagent, but not in the dilutions employed, nor do they interfere with the formation of the characteristic choline periodide crystals under these conditions. The importance of this specificity of the method will be apparent from the results to be described.

Manometric experiments

The effect of addition of choline on the oxygen uptake of rat liver extract prepared as previously described, is shown by the curves of Fig. 1. In this experiment, liver extract equivalent to 0·25 g. liver was used in each vessel. The choline solutions were placed in the side-tubes of the Warburg vessels and tipped into the main vessels immediately after the initial readings had been

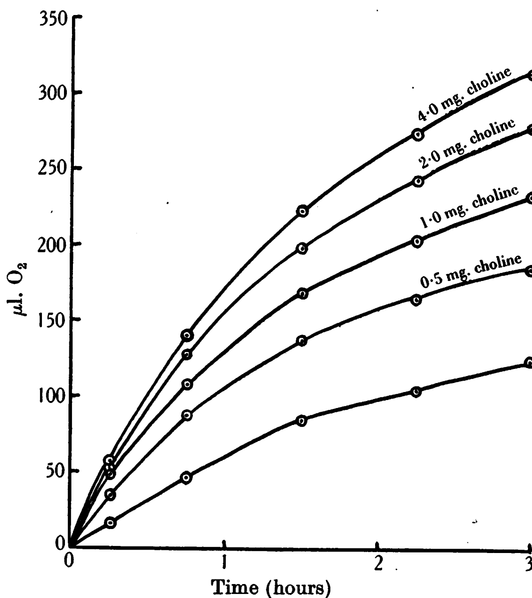

Fig. 1.

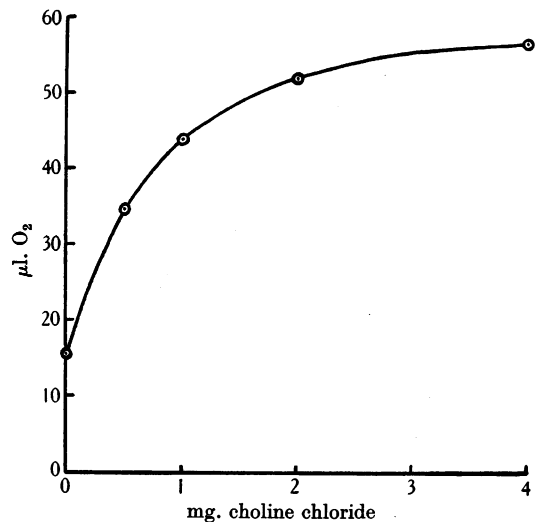

Fig. 2.

taken. It will be seen that a large increase in the rate of oxygen uptake is produced by the addition of choline. This may be due either to the oxidation of the added choline or to an activation of some oxidizing system in the extract. As will be shown later, the former explanation is the correct one, and the increased oxygen uptake is accompanied by oxidative disappearance of choline. When the values for the oxygen uptake in the first 15 min. are plotted against the amount of choline chloride added, the curve of Fig. 2 is obtained. This shows that the maximum effect is exerted when 4 mg. choline chloride are present in each vessel, or, since the total volume of medium in each vessel is 3 ml., when the concentration of choline chloride is of the order of 1 mg. per ml. Similar effects are observed with liver slices. Here, however, the relative increase in the respiration caused by the addition of choline is not so great, presumably because

the rate of oxygen uptake of a liver slice is much higher than that of an equivalent amount of extract.

The fact that the increase in oxygen uptake is due to oxidation of the added choline is apparent from the results of the following three large-scale experiments which were carried out during attempts to isolate the oxidation product.

In each of the experiments 144 mg. choline chloride were used, which were divided between nine Warburg vessels—16 mg. in each.

Rat liver extract was used for the oxidation, extract equivalent to 1 g. liver being placed in each vessel. The oxygen uptake of the tissue extract alone was measured and also that of the tissue extract with added choline. The experiments were of 3 hr. duration. At the conclusion of this time the experimental fluids from the vessels in which choline had been present were united, the protein was precipitated with an equal volume of 20 % trichloroacetic acid and the remaining choline was estimated both by the periodide and by the reineckate methods. Since the increase in oxygen uptake, due to the presence of choline, was known, the volume of oxygen required for the oxidation of 1 mg. choline chloride could be calculated.

The results obtained are shown in the following table.

		Choline remaining				
Exp.	Choline added mg.	Periodide method mg.	Reineckate method mg.	Choline oxidized mg.	Extra oxygen uptake µl.	Oxygen uptake per mg. choline chloride disappearing µl.
1	144	55	144	89	8245	92·6
2	144	47	138	97	8502	87·6
3	144	55	141	89	8065	90·6

It will be observed that according to the periodide method of estimation, the increase in oxygen uptake produced by the addition of choline is accompanied by disappearance of choline, about 90 µl. extra oxygen being taken up for every mg. choline chloride disappearing. According to the reineckate method of estimation however, there is little disappearance of choline. This suggests that the choline is oxidized to a compound which gives an insoluble reineckate but which does not react with Florence's reagent to give an insoluble periodide resembling choline periodide in crystalline form.

If during the reaction 1 mol. of choline combines with 1 atom of oxygen the increased oxygen uptake per mg. choline chloride disappearing should be 80 µl. The figure obtained in the three experiments described above is about 10 % larger than this. Bernheim & Bernheim [1933] came to the conclusion that choline is oxidized by liver extract with the consumption of $1\frac{1}{2}$ atoms of oxygen per mol. of choline, and that the reaction taking place was therefore of a complex type involving condensation. Trowell [1935], using washed liver pulp, obtained results more in agreement with those reported here. The results of 6 experiments in which 0·4 mg. choline chloride was added to washed liver pulp, gave an average increase in oxygen uptake of 38 µl. or 95 µl. per mg. choline chloride.

As a working hypothesis, it may be assumed that in the oxidation 1 mol. of choline combines with 1 atom of oxygen. If this is the case, the reaction which appears most probable is oxidation of choline at the alcoholic group with formation of betaine aldehyde.

The effect of cyanide

Bernheim & Bernheim [1933] stated that although the oxygen uptake of liver extract itself is almost completely inhibited by $M/200$ cyanide, the oxidation of choline is unaffected. In a reinvestigation of the effect of cyanide, a marked inhibitory action on choline oxidation has been found, even with much lower concentrations of cyanide than $M/200$. The results of one experiment are shown in Fig. 3. Rat liver extract equivalent to 0·25 g. fresh liver was used in each

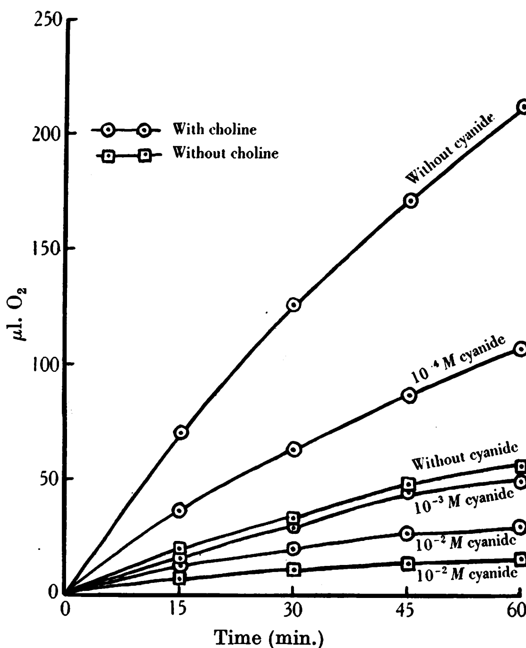

Fig. 3.

vessel and the effect of different concentrations of cyanide was observed both on the oxygen uptake of the extract alone and on that of the extract in presence of 5 mg. choline. The inner cups contained the appropriate potash-cyanide mixture to prevent change in the concentration of cyanide in the reaction mixture during the course of the reaction, according to Krebs [1935]. It will be seen that a concentration of cyanide as low as 10^{-4} M produces an appreciable inhibition of choline oxidation, while 10^{-3} M cyanide reduces the oxygen uptake to a value below that of the tissue extract without added choline. It was thought possible that the difference between these results and those of other workers might be due to the fact that the choline was placed in the side-tubes of the Warburg vessels and was not tipped into the main vessel until after the initial readings had been taken. The inhibition might then be an irreversible one similar to that observed by Dixon & Keilin [1936] when xanthine oxidase was incubated in absence of substrate. However, similar inhibitions were obtained when the choline was added to the liver extract before the cyanide. Moreover, in experiments where alkali alone was used in the inner cup to absorb CO_2, a diminution of the inhibition was observed, as the cyanide was absorbed by the alkali, showing that the inhibition produced is a reversible one.

IDENTIFICATION OF THE OXIDATION PRODUCT

For identification of the product of oxidation it was ultimately found necessary to work on a large scale. Details of one such large-scale experiment are given here.

Six rat livers of total weight 32·9 g. were ground with sand in a mortar and extracted with 50 ml. *M*/5 phosphate buffer *p*H 7·4. The mixture was allowed to stand for 15 min. at room temperature and was then squeezed through muslin. The resulting suspension diluted to 150 ml. was placed in a 500 ml. flask, together with 600 mg. choline chloride. The flask was then filled with oxygen and was mechanically agitated in a water-bath at 37° for a period of 3 hr. The suspension was reoxygenated several times during the course of the experiment to maintain aerobic conditions. After 3 hr. 150 ml. 20 % trichloroacetic acid were added and the precipitated protein was filtered off. Estimation of choline in the filtrate by the periodide method showed that 106 mg. choline remained unoxidized. To the combined filtrate and washings excess saturated ammonium reineckate solution was added and the mixture was cooled in ice before filtering off the precipitate which was washed with ice-cold water and dried *in vacuo*; wt. 1·7275 g.

The product so obtained consisted of a mixture of the reineckates of choline and of its oxidation product. For purification the reineckate was first decomposed according to the method of Kapfhammer & Bischoff [1930]. For this purpose it was dissolved in a mixture of acetone and water, and saturated silver sulphate solution was added until precipitation of silver reineckate was complete. The filtrate and washings were evaporated to a small bulk under reduced pressure and any excess silver and sulphates were removed by the addition of barium chloride solution. Finally any traces of barium were removed by addition of dilute sulphuric acid. The combined filtrate and washings were mixed with 10 ml. of a saturated aqueous solution of picric acid and concentrated under reduced pressure until crystals began to appear. The solution was placed in the refrigerator overnight and the crystals of choline picrate then filtered off. From the filtrate the unknown oxidation product was precipitated as the reineckate, which was washed in ice-cold water and dried *in vacuo*; wt. 1·0182 g. A portion of the reineckate so obtained was recrystallized first from large volumes of warm 3 % HCl and then from warm water. The product so obtained melted over the range 255–260° on rapid heating.

When the purified reineckate was decomposed in the usual way with silver sulphate, followed by treatment with barium chloride and dilute sulphuric acid, and the resultant solution was concentrated to a small volume under reduced pressure and finally taken to dryness in a vacuum desiccator a gummy crystalline mass was obtained which was very deliquescent. Attempts to recrystallize did not yield a product suitable for analysis. It was found, however, that the substance reacted as an aldehyde, giving a silver mirror with ammoniacal silver nitrate solution. This strengthened the impression already gained from a study of the oxygen uptake that it might be betaine aldehyde. Some confirmation of this results from the analysis of the reineckate. (Found (Weiler): C, 25·81, 25·93; H, 4·32, 4·66 %. Betaine aldehyde reineckate $C_9H_{18}ON_7S_4Cr$ requires C, 25·72; H, 4·29 %.) A portion of the purified reineckate was decomposed and the unknown compound was precipitated as the aurichloride. After recrystallization from 1 % HCl containing a trace of gold chloride this melted at 215–217°. Further recrystallization produced no change in the melting point. (Found (Weiler): C, 13·81; H, 2·97; N, 3·21 %. Betaine aldehyde aurichloride

$C_5H_{12}ONAuCl_4$ requires C, 13·61; H, 2·72; N, 3·17%.) The analysis of the reineckate and the aurichloride therefore suggest that the compound is betaine aldehyde.

Attempts were made to prepare the 2:4-dinitrophenylhydrazone, but the latter appeared to be too soluble to allow its isolation in a pure state to be readily attainable. No precipitate was formed when the 2:4-dinitrophenylhydrazine reagent was mixed with a solution of the unknown compound, even after prolonged standing at room temperature. Attempts to separate a hydrazone by concentration of the solution did not yield a pure product. However, when the reaction mixture was made alkaline, a deep red coloration was obtained, indicating that a hydrazone was present. This observation was utilized later to follow the oxidation of choline by tissues.

Oxidation with silver oxide

Fischer [1893; 1894], who synthesized betaine aldehyde, showed that by treatment with silver oxide it is converted into betaine which he isolated as the aurichloride. The action of silver oxide on the unknown compound was therefore studied.

0·3 g. of the purified reineckate was decomposed in the usual way, with silver sulphate, followed by treatment with barium chloride and dilute sulphuric acid. The final filtrate was concentrated under reduced pressure to a volume of about 10 ml. This was then shaken with a suspension of freshly precipitated silver oxide until a drop of the filtrate showed no reducing action towards warm ammoniacal silver nitrate solution. The filtrate was acidified with a little dilute HCl, 10 ml. of 2% gold chloride solution were added, and the mixture was concentrated under reduced pressure until crystal formation began. The solution was placed in the refrigerator overnight and the crystals were then filtered off, washed with a little ice-cold water and dried *in vacuo*; wt. 0·2034 g.; M.P. 248–250° on rapid heating. On recrystallization from 0·5% HCl containing a few drops of 2% gold chloride solution no change in melting-point occurred. A sample of authentic betaine aurichloride was also prepared which melted at 249–250°; mixed M.P. 248–250°. It may therefore be concluded that on oxidation with silver oxide the unknown compound is converted to betaine.

Oxidation with permanganate

Oxidation with potassium permanganate in acid solution also gives betaine, but this reaction is of less value than the silver oxide oxidation as a proof of constitution, since, as shown by Lintzel & Fomin [1931] both choline and betaine aldehyde are converted into betaine by permanganate. In this case the betaine was isolated as the reineckate, M.P. 153–155° on rapid heating. A sample of the authentic betaine reineckate melted at 154–156°; mixed M.P. 153–155°. This reineckate is soluble in alkaline solution and insoluble in acid solution, in contrast to the choline reineckate which is insoluble in both acid and alkaline solutions. This difference in solubility was used by Strack & Schwaneberg [1936] for separation of betaine from choline.

The unknown compound therefore is formed by oxidation of choline, the oxygen uptake is in the neighbourhood of 1 atom of oxygen per molecule of choline oxidized, the product has the properties of an aldehyde, and by further oxidation with silver oxide or with permanganate in acid solution it is converted into betaine. The evidence is therefore sufficient to identify the compound as betaine aldehyde.

Discussion

The increase in oxygen uptake which occurs when choline is added to respiring rat liver slices or extract, is due to oxidation of choline to betaine aldehyde. It is not yet known whether the enzyme responsible is specific for choline oxidation. Preliminary investigations with inhibitors suggest that the enzyme is not identical with amino-acid oxidase or alcohol dehydrogenase, since neither glycine nor ethyl alcohol inhibits the oxidation. On the other hand, inhibition of the oxidation is produced by ammonium chloride and sulphate, trimethylamine and betaine. The properties and distribution of the enzyme will be dealt with in a later publication. It may be mentioned here that Bernheim & Bernheim [1933] concluded that oxidation of choline was brought about by rat and cat liver and kidney tissue but not by guinea-pig liver. We have also found that rat liver and kidney are active, but that guinea-pig liver and kidney are apparently inactive.

A further point to be investigated is the significance of betaine aldehyde in animal metabolism. Does the reaction merely constitute the first stage in the removal of choline, or is it connected in any way either with the formation of acetylcholine or with the action of choline in the prevention and cure of fatty livers? With regard to acetylcholine formation it is of interest to recall that Strack *et al.* [1935] suggested that this compound might arise as a result of a Cannizzaro reaction between betaine aldehyde and acetaldehyde, while by aldol condensation of the same two substances, carnitine aldehyde would be produced which on oxidation would give carnitine. These possibilities are under investigation.

There appears to be no obvious connexion between the oxidation of choline to betaine aldehyde and the action of choline on fatty livers. Trowell [1935], indeed, came to the conclusion that the addition of choline to rat liver slices caused an inhibition of their fat oxidation. This conclusion was based in part on the observation that the increased oxygen uptake produced by the addition of choline was always less than that required for the oxidation of all the choline added, assuming that 1 mol. of choline requires 1 atom of oxygen. Trowell, however, used concentrations of choline as high as 2·3 %, i.e. about 70 mg. in each manometric vessel. The duration of the experiments after the addition of choline was only 1 hr. Under such conditions only a small proportion of the choline could be oxidized during the experimental period. In cases where low concentrations of choline were used it should be noted that the velocity of choline oxidation falls rapidly as the choline concentration falls below that required for the saturation of the enzyme. Hence, here also, the complete oxidation of the added choline would be improbable.

In the experiments reported in the present work the choline was generally maintained throughout the experiments in concentration sufficient to keep the enzyme saturated. Under these conditions the increased oxygen uptake is, as already described, about 10 % more than the theoretical value, assuming that the molecule of choline takes up 1 atom of oxygen. This result was obtained using liver extract for the oxidation. On the other hand, where a low concentration of choline is employed and the enzyme is in excess, so that complete oxidation of all the choline should occur during the experimental period, the increased oxygen uptake according to Bernheim & Bernheim [1933] corresponds to $1\frac{1}{2}$ atoms per mol. of choline. In a preliminary investigation of the apparent discrepancy we have found that under such conditions the oxygen uptake may attain values nearly equivalent to 1 mol. of oxygen per mol. of choline. This appears to be due to the further oxidation of the betaine aldehyde, since the latter, when added to

rat liver and kidney slices and extracts, apparently produces a small increase in the rate of oxygen uptake which is accompanied by the disappearance of the aldehyde. Some evidence has been obtained that the oxidation product is betaine. This will be dealt with in a later publication.

Summary

1. The action of choline in increasing the rate of respiration of rat liver slices or extracts has been investigated.

2. The increase in respiration rate is accompanied by disappearance of choline, indicating that the effect is due to oxidation of choline.

3. When the concentration of choline is maintained at the optimum throughout the experimental period and liver extract is used for the oxidation, the extra oxygen per mg. choline disappearing is about 10% higher than the theoretical value, assuming that 1 mol. combines with 1 atom of oxygen.

4. The isolation of the oxidation product in the form of its reineckate and its aurichloride is described. The compound gives the reactions of an aldehyde and by further oxidation with silver oxide or with permanganate in acid solution it is converted into betaine. In view of these facts and the results of analyses of the reineckate and the aurichloride it is identified as betaine aldehyde.

5. The oxidation is inhibited by low concentrations of cyanide. This inhibition is reversible.

6. The nature of the enzyme responsible for the oxidation is discussed and also the possible significance of betaine aldehyde in animal metabolism. Some evidence is put forward which suggests that rat liver and kidney oxidize it slowly to betaine.

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