terol was subjected to a second saponification with 20% aqueous potassium hydroxide for 60 hours. Repetition of the ion exchange separation still gave some neutral material, which did not contain a digitonin precipitable component. The specific activities of the acids obtained upon elution corresponded closely with the activities of the acids obtained after the first saponification. In the cases of yolks 1, 2 and 7, where a second saponification was carried out, the specific activities were $7.37 \times 10^{-4}$ μc/mg, $2.60 \times 10^{-3}$ μc/mg, and $2.61 \times 10^{-2}$ μc/mg, respectively.

*Glycerol.* The aqueous solution remaining after the saponification mixture had been extracted was distilled to dryness at reduced pressure. In several cases, the salt residue was extracted with hot pyridine and, after cooling, benzoyl chloride was added to this solution. Acidification and ether extraction yielded the glycerol tribenzoates of yolks 1, 2, 3, 4 and 6. These derivatives had melting points in the range 73-76° and none depressed the melting point of an authentic sample of this material.‡ For yolks 5, 7, 8, 9 and 10, the salt residue was extracted with hot acetone. Evaporation of the acetone in a current of nitrogen left the desired glycerol, which was also converted to the tribenzoate in a few cases. Wet combustion was used to assay radioactivity. In cases where a sample was assayed both as free glycerol and as tribenzoate the specific activities were in agreement.

*Summary.* (1) The distribution of radioactivity in the yolks of eggs laid by a hen fed sodium acetate-1-$C^{14}$ has been determined. (2) The radioactivity appears in all fractions of all yolks in the following order of increasing specific activity: protein, glycerol, phospholipids, fatty acids, cholesterol.

The interest of Prof. Melvin Calvin during the course of this investigation is gratefully acknowledged.

‡ We are indebted to Prof. James Cason for supplying a sample of this material.

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## The Mechanisms of the Choline Oxidase and Transmethylase Enzyme Systems.* (19021)

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In an earlier investigation of the choline oxidase system(1) various requirements of the enzyme system for maximum activity in rat liver homogenates were studied. Since the work of Muntz(2) implied that in order for transmethylation from choline to homocysteine to take place choline must first be converted to betaine, an investigation of the role of all 3 possible donors, *i.e.*, choline, betaine aldehyde, and betaine, in forming methionine from homocysteine was undertaken.

Since previous work concerning transmethylation to homocysteine was carried out anaerobically(2,3), it appeared desirable to compare the roles of choline and its oxidation products in transmethylation both aerobically and anaerobically and to correlate oxidation data with transmethylation data. In the earlier study of the choline oxidase system(1), the results indicated that the oxidation of choline to betaine did not occur as smoothly as might be expected since after the first 10

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minutes of choline oxidation betaine aldehyde oxidase became the rate-limiting enzyme in the system. Therefore, oxygen uptake data for oxidation of choline did not necessarily indicate that a mole of betaine was being formed for each mole of oxygen taken up. In order to study the actual mechanism of the reactions, the formation of betaine could be estimated indirectly by using a bicarbonate buffer and measuring acid production manometrically, provided suitable controls were employed. In this way the oxidation of choline and betaine aldehyde could be correlated with the formation of betaine and also with transmethylation if homocysteine was included in the system.

*Experimental.* Adult male Holtzman rats weighing from 200 to 250 g were used as experimental animals. At the time of experiment the animals were sacrificed by decapitation, the livers removed *in toto,* and chilled in cracked ice. A portion of the liver was weighed out and homogenized in 5 volumes of ice-cold pH 7.8 bicarbonate buffer of the following composition: 0.039 M sodium bicarbonate and potassium chloride and 0.0033 M magnesium sulfate. One ml of homogenate was pipetted into previously prepared Warburg vessels. In the side arm of each of 16 Warburg vessels was added 0.5 ml 0.0234 M DL-homocysteine hydrochloride (General Biochemicals, Inc.) prepared in the bicarbonate buffer described above. 0.1 ml of water was added to the side arms of flasks 1, 2, 9, and 10. To the side arms of flasks 3, 4, 11, and 12 was added 0.1 ml 0.128 M choline chloride; of flasks 5, 6, 13, and 14, 0.1 ml of 0.128 M betaine aldehyde, prepared according to Voet (4); of flasks 7, 8, 15, and 16, 0.1 ml of 0.128 M neutralized betaine hydrochloride. The first 8 flasks were employed both for measuring oxidation of substrates and for determining carbon dioxide evolution due to betaine formation from choline and betaine aldehyde by the "direct method"(5). Therefore, 4 alternate flasks of the first 8 contained 0.2 ml 10% potassium hydroxide and the remaining 4 contained 0.2 ml water in the center wells. The last 8 flasks were employed for determining whether betaine could be formed anaerobically from either choline or betaine aldehyde. Since homocysteine can be desulfurated by rat liver homogenates with

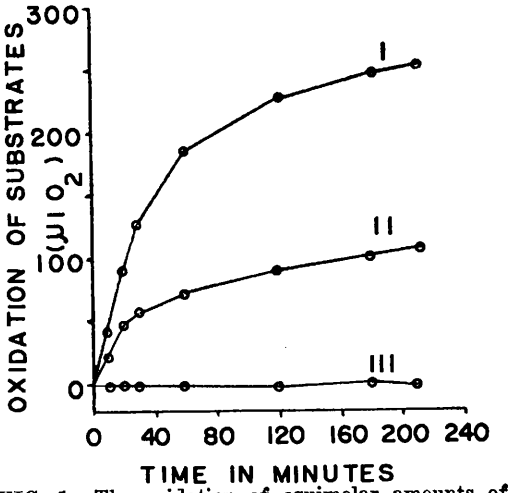


FIG. 1. The oxidation of equimolar amounts of choline (Curve I), betaine aldehyde (Curve II), and betaine (Curve III) by rat liver homogenate.

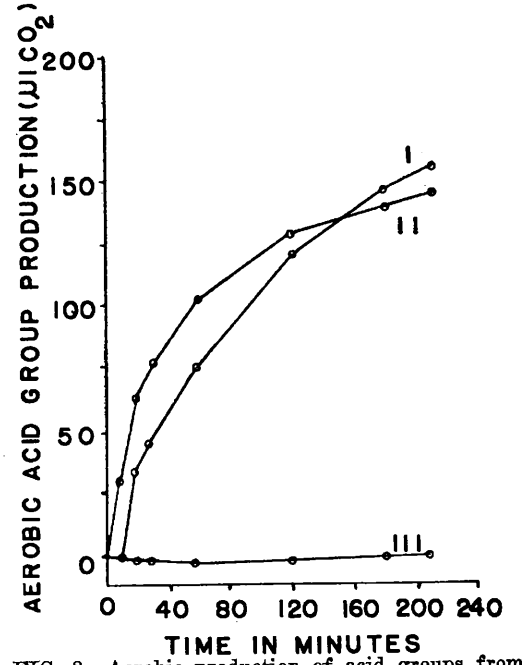


FIG. 2. Aerobic production of acid groups from choline (Curve I), betaine aldehyde (Curve II), and betaine (Curve III) by rat liver homogenate.

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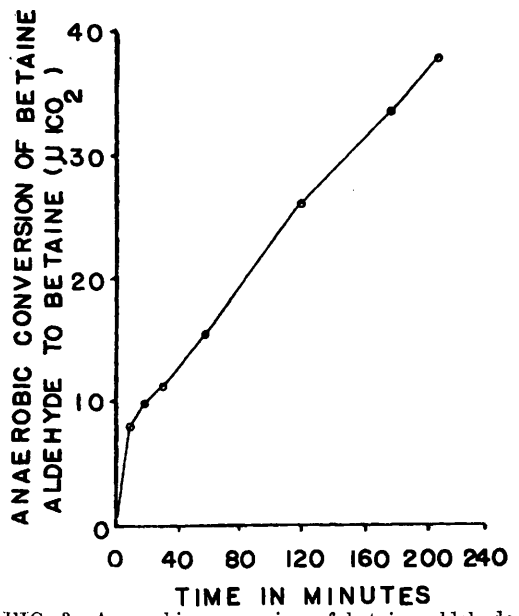


FIG. 3. Anaerobic conversion of betaine aldehyde to betaine by rat liver homogenate.

the formation of hydrogen sulfide(2), 0.2 ml of 10% cupric sulfate penta hydrate in 1 N sulfuric acid was added to the center wells of the last 8 flasks to absorb any hydrogen sulfide that might be liberated.

After the liver homogenate was added to the main compartment of the flasks, the last 8 flasks were gassed for 10 minutes with 95% nitrogen-5% carbon dioxide mixture. All flasks were then equilibrated on the Warburg bath at 37°C for 10 minutes after which the contents of the side arms were added to the homogenate and manometer readings taken at intervals for 210 minutes. The methionine present in each flask after the 210-minute period was estimated according to the method outlined by Dubnoff(3).

*Results. Manometric studies.* The data obtained manometrically have been analyzed as shown in Fig. 1 to 3 and Table I. The data presented are an average of the results from 10 animals although each animal conformed to the same pattern. In Fig. 1 the oxygen uptake data reveals that at the end of 210 minutes the oxidation of choline and betaine aldehyde approached the theoretical values of 286 and 143 μl $O_2$, respectively, assuming that the oxidation of choline to betaine requires 1 mole of oxygen and that of betaine aldehyde, 0.5 mole of oxygen. The oxidation of betaine was zero, which also implies that the methionine formed from homocysteine was not oxidized in the system. In Fig. 2 the calculated carbon dioxide evolution due to formation of acid (assumed to be betaine) from the substrates added indicates that betaine aldehyde was oxidized directly to betaine as shown by the absence of a lag in the curve. However, for the first 10 minutes choline did not give rise to betaine as shown by the lack of acid production for the first 10-minute period. After 210 minutes the total acid production both from choline and betaine aldehyde approached approximately the same value. However, the actual amount of betaine formed from both substrates as measured by carbon dioxide production was only one-half the theoretical value (286 μl $CO_2$). Therefore, comparing Fig. 1 and 2, the carbon dioxide evolution from choline should equal the oxygen uptake and that from betaine aldehyde should be twice the oxygen uptake, assuming the following reactions are the only ones involved:

$$\text{Choline} \xrightarrow{+\frac{1}{2}O_2} \text{betaine aldehyde}$$

$$\xrightarrow{+\frac{1}{2}O_2} \text{betaine (1 mole choline + 1 mole } O_2 \longrightarrow \text{1 mole betaine, or 1 } CO_2\text{; 1 mole betaine aldehyde + } \tfrac{1}{2} \text{ mole } O_2 \longrightarrow \text{1 mole betaine, or 1 } CO_2\text{).}$$

In the anaerobic experiments no measurable production of betaine from choline was

TABLE I. Ratio of Carbon Dioxide Evolution to Oxygen Consumption (Betaine Formation to Substrate Oxidized).

| | $CO_2 : O_2$ | | | |
|---|---|---|---|---|
| | Choline | | Betaine aldehyde | |
| Time (min) | Uncorrected* | Corrected* | Uncorrected* | Corrected* |
| 10 | 0 | 0 | 1.50 | 1.10 |
| 20 | .41 | .29 | 1.35 | 1.13 |
| 30 | .36 | .27 | 1.37 | 1.18 |
| 60 | .41 | .33 | 1.44 | 1.20 |
| 120 | .53 | .41 | 1.37 | 1.10 |
| 180 | .59 | .46 | 1.38 | 1.05 |
| 210 | .60 | .47 | 1.34 | 1.03 |
| Theoretical ratio | 1 | | 2 | |

* See text for explanation of terms.

TABLE II. Methionine Formation and Gas Exchanges Under Aerobic and Anaerobic Conditions from Homocysteine and the Substrates Listed After 210 Minutes.

| Gas phase | Substrate | Methionine formed ($\mu$ moles) | Oxygen consumed ($\mu$ moles) | $CO_2$ evolved (betaine formed) ($\mu$ moles) |
|---|---|---|---|---|
| Air | Choline | .40 | 11.4 | 7 (5.3 corrected)* |
| | Betaine aldehyde | .40 | 4.8 | 6.4 (4.8 " )* |
| | Betaine | .40 | 0 | 0 |
| 95% $N_2$– | Choline | .10 | 0 | 0 |
| 5% $CO_2$ | Betaine aldehyde | .36 | 0 | 1.7 |
| | Betaine | 1.32 | 0 | 0 |

* See text for explanation of term.

observed as shown in Fig. 3. However, with betaine aldehyde as substrate, a marked production of betaine was observed anaerobically as demonstrated by the curve for betaine aldehyde in Fig. 3. Therefore, it appears that 2 pathways exist for conversion of betaine aldehyde to betaine, one requiring oxygen and the other proceeding anaerobically. The anaerobic pathway which may be a dismutation reaction, appears to be approximately one-third as active in rat liver homogenates as the aerobic pathway as shown by the relative rates of conversion of betaine aldehyde to betaine.

In Table I are presented the ratios of carbon dioxide evolution (or betaine formation) to oxygen uptake for choline and betaine aldehyde at the various time intervals. The ratios have been calculated in 2 ways: the ratios actually obtained and the ratios corrected for anaerobic conversion of betaine aldehyde to betaine assuming that the anaerobic pathway is still active under aerobic conditions. The latter method is probably more meaningful since the carbon dioxide generated by the aerobic conversion of the substrate to betaine should be compared to the oxygen uptake involved in conversion. The calculations indicate, therefore, that the ratios for choline approach 0.50 and for betaine aldehyde 1.00, both of which are one-half the theoretical ratios. Therefore, only one-half as much betaine is formed from either substrate as expected. The reason for the low yield of betaine from both choline and betaine aldehyde is not clear although one might surmise several possible explanations. One of them which would fit the data obtained assumes an instantaneous dimerization of betaine aldehyde at the pH employed, 7.8.

*Transmethylation studies.* The results of the methionine analyses after the 210-minute incubation period are presented in Table II. In order to compare the manometric results with formation of methionine, the final oxygen uptake and carbon dioxide evolution data are reported in the table as micromoles of oxygen consumed or carbon dioxide evolved and the methionine production as micromoles of methionine formed. It can be observed from the table that aerobically choline, betaine aldehyde, and betaine are equivalent in transfer of a methyl group to homocysteine. Also there appears to be no correlation either with the oxidation of substrates or with the formation of betaine since aerobically a large excess of both choline and betaine aldehyde are oxidized to betaine over the amount actually involved in transmethylation. That the methionine formed is not oxidatively removed is borne out by the fact that no oxygen uptake was observed in the flask containing betaine as substrate (Fig. 1) even though methionine was formed.

The anaerobic results indicate that methyl donation by choline occurs only to a negligible extent, which is probably due to lack of conversion of choline to betaine. However, betaine aldehyde, since it is anaerobically converted to betaine, does contribute methyl groups to homocysteine though not as greatly as betaine itself. Moreover, the anaerobic transfer of methyl groups from betaine is significantly greater than the aerobic transfer.

*Summary.* (1) The mechanism of choline and betaine aldehyde conversion to betaine and methyl group transfer to homocysteine have been investigated both in aerobic and anaerobic systems. (2) The expected yield of acid groups, assumed to be betaine, from

oxidation of choline and betaine aldehyde is only one-half the theoretical. (3) Betaine aldehyde appears to be metabolized by 2 pathways, one an aerobic oxidation and the other an anaerobic conversion to betaine. (4) Choline, betaine aldehyde, and betaine are equivalent methyl group donors to homocysteine under aerobic conditions. Anaerobically choline is almost inactive in furnishing methyl groups to homocysteine. Betaine, however, is a more active methyl donor anaerobically than under aerobic conditions.

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## Effect of Aminopterin on Choline, Betaine Aldehyde, and Betaine Metabolism.* (19022)

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In several recent publications(1-3) it has been reported that dietary aminopterin causes a marked decrease in tissue choline oxidase activity. This decrease in activity has been reversed by incubating the liver homogenates with combinations of several of the presumed precursors of the *Leuconostoc citrovorum* (LCF) factor, namely, ascorbic acid, folic acid, vit. $B_{12}$, and LCF itself(1,2). Moreover, it has been shown from this laboratory that dietary aminopterin induces a fault in the ability of rats to synthesize ascorbic acid(4). The sum of the preceding work, therefore, indicates that folic acid and ascorbic acid metabolism are disturbed by dietary aminopterin, and that this may be the basic cause of the decrease in choline oxidase activity. Since the choline oxidase(5) and betaine aldehyde oxidase(6) systems are believed to be intimately connected with the transmethylation reactions of homocysteine, and since a deficiency of dietary folic acid has been shown to disturb this transmethylation reaction in chickens(7), the present investigation was undertaken to determine the extent of the disturbance of the transmethylation reactions involving choline, betaine aldehyde and betaine by dietary aminopterin in rats.

In a preceding publication(6), a study of the mechanism of the metabolism of choline and betaine aldehyde has shown that the latter metabolite can be converted to betaine either aerobically or anaerobically, while choline oxidase is entirely an oxygen-requiring system. Because of the close relationships of these metabolites with transmethylation, it appeared desirable to study the effects of dietary aminopterin on the different aspects of choline, betaine aldehyde, betaine, and methionine metabolism uncovered in the preceding investigation.

*Experimental and results.* Adult male rats of the Holtzman strain were employed as experimental animals. One group of rats was fed a normal synthetic ration containing 18% casein as previously described(1). Other animals were fed the same ration except that 4 mg of aminopterin† were included per kilo

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