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An In-Vitro Study of Transmethylation.

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An In-Vitro Study of Transmethylation

by

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INTRODUCTION

It has been observed that rats and other animals which are kept on a diet deficient in choline, are retarded in their growth and develop a condition known as fatty infiltration of the liver. It has also been demonstrated that choline will support growth of rats kept on a cystine-methionine-free diet, supplemented with homocystine. It has been concluded, therefore, that choline is necessary in the diet of the rat and the suggestion has been made that the animal organism can transfer a methyl group from choline to homocystine in order to synthesize methionine.

(1) $2\ -CH_3$ + $(SCH_2CH_2CHNH_2COOH)_2$ ⟶ 2 $CH_3SCH_2CH_2CHNH_2COOH$

from choline homocystine

Tracer experiments support this view that the methyl group is transferred in toto. It has been shown furthermore that the animal is incapable of synthesizing methyl groups, and that the sources of these labile methyl groups are primarily choline, methionine, and betaine.

The term "transmethylation" is applied to this ability of the animal to transfer methyl groups from one compound to another. In-vitro experiments have shown that the biosynthesis of creatine also occurs by transmethylation, and that the process is an enzymatic one, being restricted to the liver and kidney of animals. The biosynthesis of methionine from choline or betaine and homocysteine has been demonstrated

also by in-vitro experiments with animal tissue.

Of all transmethylation reactions which have been demonstrated to occur by feeding and isotopic experiments, the biosynthesis of choline has not been isolated by in-vitro experiments. Within the last five years two research workers in independent experiments claim that they have been able to demonstrate by in-vitro experiments the reaction between methionine and ethanolamine to produce choline. These workers also claimed that this enzymatic process is not restricted to the animal liver but occurs in animal muscle and in etiolated wheat germs.

Due to the importance of the biosynthesis of choline, it seemed advisable to re-investigate the various claims and to attempt to repeat the work. After the reaction had been definitely established, an attempt would then be made to study the enzyme or enzyme system responsible for the reaction. On the other hand, if attempts were unsuccessful to prove by in-vitro experiments that a reaction between methionine and ethanolamine took place, it would then be of paramount importance to study the fate of methionine in the animal body.

HISTORICAL

The first reported observation that the animal body is capable of transferring methyl groups was made by His[1] in 1887. His observed the excretion of methylpyridylammonium hydroxide in the urine of dogs after they had been fed with pyridine. His raised the question as to how the methyl group was transferred to pyridine. Was it possible that the methyl group had been carried by an intermediate, like the iodide in methyl iodide?

Hofmeister[2] in 1894 reported that the liver, lungs, and testicles of dogs readily converted inorganic compounds of selenium and tellurium into odorous substances, presumably dimethyl selenide and dimethyl telluride. Heating the tissue to 40 - 50°C, treatment with acids, alkalis, or alcohol destroyed this "methylating" ability. Hofmeister, therefore, concluded that this process was enzymatic, because the rigid treatment would have inactivated almost any enzyme.

It was not until 1939 that du Vigneaud[3] presented concrete evidence that the animal organism was capable of transferring methyl groups from one compound to another in the biosynthesis of vital growth factors. In feeding experiments du Vigneaud was able to show that choline would support growth of rats kept on a cystine-methionine-free diet, supplemented with homocystine. These same animals on a choline-deficient diet were greatly retarded in their growth and developed fatty infiltration of the liver. Rose[4] included methionine in the list of "indispensable" amino acids, those amino acids, essential for normal growth of the animal, which

could not be totally synthesized by the animal organism without the necessary intermediates. Therefore, du Vigneaud concluded that on the basis of his experiments the animal organism had transferred a methyl group from choline to homocystine in order to synthesize methionine.

Du Vigneaud has summarized his early work from 1939-1941 in a review article[5]. Homocystine will support growth in the rat on a methionine-free diet only in the presence of choline and betaine. The conclusion drawn, was that the animal organism was incapable of generating methyl groups, and that methyl groups in a utilizable form were essential in the diet. If the growth effect had been due solely to prevention of fatty infiltration of the liver, then triethylcholine which also prevents fatty infiltration, would have supported growth. However, triethylcholine retarded growth of the rat on a methionine-free diet.

Direct proof for this methyl transfer was offered by du Vigneaud by labeling the methyl groups of methionine and choline with deuterium. On feeding rats deuteriocholine, the formation of deuteriomethionine was demonstrated. Furthermore when deuteriomethionine was fed to the animals, choline and creatine containing deuterium in their methyl groups, were isolated from the carcass. Here then was proof for two more transmethylating reactions, a transfer of the methyl group of methionine to form creatine and choline. These three reactions can be summarized as follows without postulating any precursors or by-products.

(2.) $(CD_3)_3\overset{\displaystyle OH}{\overset{|}{N}}CH_2CH_2OH \longrightarrow CD_3SCH_2CH_2CH(NH_2)COOH$

choline — methionine

(3.) $CD_3SCH_2CH_2CH(NH_2)COOH \longrightarrow C(=NH)(NH_2)(NCH_3COOH)$

methionine — creatine

(4.) $CD_3SCH_2CH_2CH(NH_2)COOH \longrightarrow (CD_3)_3\overset{\displaystyle OH}{\overset{|}{N}}CH_2CH_2OH$

methionine — choline

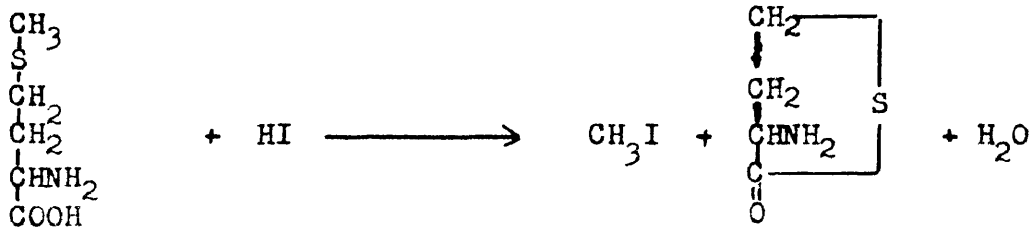
It has thus been demonstrated that transmethylation is responsible for the biosynthesis of methionine, creatine, and choline. Renewed interest has been shown in studying the reaction which His[1] had observed many years before, the methylation of pyridine. Several investigators have been able to demonstrate that the methylation of nicotinamide[6,7] and noradrenaline[8] are enzymatic processes.

Biosynthesis of Methionine. Homocystine will support the growth of rats on a methionine-free diet only in the presence of choline and betaine.[5,9] Chandler and du Vigneaud showed that choline was a more effective methyl donor than betaine, as shown by the development of these rats on a thoroughly controlled diet. When the animals were fed homocystine on a choline-methionine-free diet, they were unable to survive.

In 1943 du Vigneaud and co-workers[10] established proof of this transmethylation reaction by incorporating deuterium into the choline of the diet of

the test animals. Choline, whose methyl groups were tagged by deuterium, was fed to rats on a methionine-deficient diet. The butyl alcohol soluble fraction of the hydrolyzed proteins was treated with HI in order to liberate CH_3I from methionine by the following reaction:

(5.)



methionine thiolactone

The methyl iodide was then converted to tetramethyl ammonium iodide. Tetramethyl ammonium iodide and its chloroplatinate derivative were analyzed for deuterium and found to contain some isotopic hydrogen. These experiments, therefore, bore out the prediction that the methyl group of methionine had originated from choline.

Borsook and Dubnoff[11] in 1947 showed by isolated in-vitro experiments that methionine could be synthesized in the rat liver by transmethylation. Rat liver slices were incubated with homocysteine, homocystine, and homocysteinethiolactone. Both choline and betaine were able to transfer their methyl groups to these methyl acceptors and synthesize methionine. The methionine was analyzed by the method of McCarthy and Sullivan[12]. The rate of the reaction was shown to be greatest with homocysteine and smallest with homocystine. The reaction was faster with betaine than with choline. The reaction was shown to be independent of oxygen, and the catalytic activity was retained

in homogenized and lyophilized rat liver.

Dubnoff in 1949[13] had to modify his earlier conclusions on the influence of oxygen on the reaction. Methylation of homocysteine in the presence of betaine actually was found to be relatively independent of oxygen tension. The methylation of homocysteine by choline approaches the rate of betaine under aerobic conditions, however, the methylation is small or negligible, anaerobically. The degree of utilization of choline methyl for methionine synthesis paralleled the activity of the enzyme choline oxidase. This enzyme catalyzes the oxidation of choline to betaine. Dubnoff observed that methionine formation did not take place in liver homogenates of animals which do not have an active choline oxidase, for example guinea pigs and rabbits. Dubnoff concluded that choline-methyl is not transferable to homocysteine in-vitro, unless the alcohol group in choline has been oxidized.

By isotope experiments Muntz[14] in 1950 confirmed Dubnoff's earlier conclusions that choline did not lose methyl groups directly but was converted to betaine before a methyl transfer could occur. Homocysteine and N^{15} labeled choline were incubated with rat liver homogenate. No dimethylethanolamine was isolated, but relatively high isotope content in dimethylglycine was observed.

Due to recent interest in the action of Vitamin B_{12}, several research workers have tried to show a connection between the action of this vitamin and the biosynthesis of methionine. Jukes[15] and co-workers demonstrated in feeding experiments with chicks that homocystine plus betaine did not promote growth of Vit B_{12}-deficient animals, but that these chicks responded to methionine alone. Jukes also showed that chicks whose diet had been supplemented with Vit B_{12},

responded to betaine plus homocystine or homocysteine. Oginsky demonstrated[16] that livers of Vit B_{12}-deficient rats exhibited a lower activity in the ability to synthesize methionine from homocystine and either choline or betaine. No decrease in choline oxidase activity was observed in these livers. Gillis and Norris[17] observed that B_{12}-deficient chicks grew as well when fed homocystine plus betaine on a methionine-deficient diet, as when comparable amounts of methionine were added. No evidence was obtained to substantiate the claim of Jukes[15] that Vit B_{12} increased the efficiency with which preformed methyl groups transfer to homocystine.

Biosynthesis of Creatine. This reaction has been most thoroughly investigated, probably due to the early development of a convenient colorimetric analysis for creatine[18]. The analysis consists of a conversion of creatine to creatinine by acid and the development of a red color in the presence of picric acid in alkaline solution. The color reaction was shown later by several investigators, who will be cited individually, to be non-specific for creatinine. It is difficult, therefore, to evaluate the work on the biosynthesis of creatine, without knowing how specific the various modifications of the original alkaline picrate method are for creatinine.

Early investigators[19,20] reported that when injecting rabbits with glycocyamine, an increase in urinary creatinine resulted. Others[21] noted an increase in creatine content of the muscle of the rabbit after injection of glycocyamine. These scientists also claimed that rabbit muscles incubated with glycocyamine showed greater creatine formation.

It was not until du Vigneaud's feeding experiments with deuterio-

methionine[22] that a reaction involving methionine in the synthesis of creatine by the animal organism could be established. Rats were fed $CD_3SCH_2CH_2CH(NH_2)COOH$ on a methionine-choline-free diet. Creatine isolated from the muscle of the carcass as the zinc chloride complex was found to contain deuterium. In order to demonstrate that deteurium occurred in the methyl group attached to the nitrogen atom, creatine was hydrolyzed to sarcosine with $Ba(OH)_2$, and the sarcosine in turn was decomposed to CH_3NH_2 with Ag_2O. The methyl amine was determined as the chloroplatinate, and deuterium was found to occur in that compound. If the rats were fed deuteriomethionine for longer periods than two months, the creatine isolated from the carcass contained more than 85% of the theoretical amount of deuterium, calculated on the basis of transfer of deuteriomethyl from dietary methionine[5]. du Vigneaud ruled out simple isotopic exchange in the animal body to account for the deuterium content in creatine; the initial deuterium concentration being such that if it had exchanged and had become mixed with ordinary hydrogen of the body, it would have been beyond limits of detection[5]. The measurement of the duteurium content was carried out by the "falling drop method" of Keston and co-workers[23].

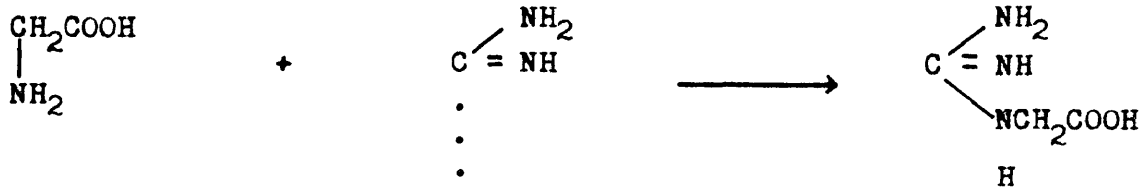
Shortly after du Vigneaud's isotope experiments had been initiated, Borsook and Dubnoff[24] demonstrated the in-vitro synthesis of creatine from methionine and glycocyamine. Liver slices of cat, rabbit, and rat were capable of converting glycocyamine to creatine, and the reaction was greatly accelerated by the addition of methionine. Creatine was determined by a modification of the method developed by Miller, Allison, and Baker[25]. The method employes the N C bacteria which adapt themselves to grow on creatine as their sole source of carbon

and nitrogen. The difference between the color developed by alkaline picrate before and after bacterial digestion, gave the true creatine value.

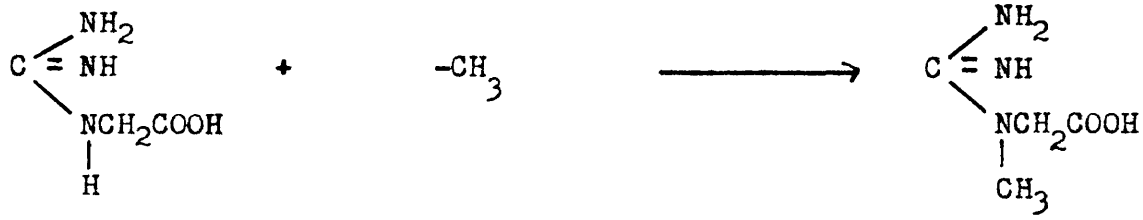
Baker and Miller[26] also demonstrated that rat kidney and liver were able to synthesize creatine in-vitro. Creatine was determined by the bacteriological method[25]. Borsook and Dubnoff[27] showed that with the exception of guinea pig and pigeon, kidney slices of all animals tested failed to methylate glycocyamine in-vitro. However, liver slices of the rat, rabbit, dog, hog, guinea pig, and pigeon were all active in this reaction. These findings indicated that creatine is normally formed by methylation of glycocyamine in the liver of animals.

The reaction of the biosynthesis of creatine was found to be irreversible, as indicated by growth studies of the rat[28]. The methyl group of creatine and creatinine is not available for the methylation of homocystine or for the synthesis of choline. Similar experiments with sarcosine resulted in the same negative findings: the methyl group of sarcosine is not available for transmethylation.

Borsook and Dubnoff[29] were able to demonstrate by in-vitro experiments that the kidney of all animals tested could form glycocyamine from glycine and arginine. Glycocyamine had been shown previously to be the precursor of creatine[24]. Bloch and Schönheimer[30] had also demonstrated by isotope experiments that the guanidine group in glycocyamine was derived from arginine, and that the third nitrogen originated from glycine. The reactions for the biosynthesis of creatine can thus be written:

(6.) 

glycine (from arginine) glycocyamine

(7.) 

glycocyamine (from methionine) creatine

Barrenscheen and Valyi-Nagy[31] claimed that they had obtained a proof for the biosynthesis of creatine from methionine and glycocyamine, using etiolated wheat extracts as a source of enzyme. Creatine was determined by the alkaline picrate method after adsorbing the sample on Frankonite -KL. The authors stated that with necessary blanks which they ran parallel to their samples, they were able to obtain good results as to the synthesis of creatine.

Other investigators[32,33] have reported that creatine is synthesized from arginine in-vitro by frog muscle brei. Menne[32] also reports that choline plus glycocyamine incubated with frog muscle, increases the yield of creatine.

G. Steensholt[34] observed creatine synthesis from methionine and glycocyamine, using minced rat muscle and liver tissue. For the creatine analysis Steensholt used the alkaline-picrate method with the following reservation:

> It is well known that the so-called Jaffe reaction . . . is not very specific. Thus guanidine acetic acid itself gives a color which may to some degree interfere with the

> creatinine determination. . . . . By working with suitable blanks, however, it is possible to eliminate the influence of interfering substances to a large extent.

The reported results by Steensholt are given in photometer readings, for example:

Sample A	Sample B
1.86, 1.90	1.51 1.56

Sample A contained glycocyamine, methionine, and tissue, whereas Sample B only contained glycocyamine and tissue.

Handler and Bernheim[35] in 1943 reported that D-methionine was about 50% as active as the natural form of the amino acid in the creatine synthesis by rat liver tissue. The keto analogue of methionine, α-keto-γ-methiolbutyric acid, was also found to be active in the transmethylation. Handler and Bernheim followed Borsook and Dubnoff's method for creatine analysis[27]. The name for the bacterial organism, which was specific for creatine, was chosen as "Corynebacterium creatinovoraus". Handler and Bernheim found the sulfoxide and sulfone of methionine to be ineffective in creatine synthesis. Valyi-Nagy[36] claimed that methionine sulfoxide was as good a methyl donor in creatine synthesis as methionine in the case of guinea pig muscle paste. He postulated that the first and obligatory step in transmethylation was an aerobic reaction, the aerobic oxidation of methionine to sulfoxide and sulfone. The actual methyl transfer from the oxidized methionine would take place in the presence or absence of molecular oxygen.

In 1945 Borsook and Dubnoff[37] showed by in-vitro experiments with rat liver slices that choline would not accelerate the methylation of

glycocyamine by itself, but that choline plus homocystine was capable of methylating glycocyamine in the biosynthesis of creatine. It was, therefore, concluded that methionine transferred its methyl group to glycocyamine.

In 1946 du Vigneaud and co-workers[38] reversed their previous findings on the availability of the methyl group of sarcosine for the biosynthesis of creatine and choline[28]. By the use of isotopic N^{15} in the sarcosine molecule in feeding experiments, it was shown that sarcosine was capable of synthesizing choline and creatine in the animal body, although at a much slower rate than that at which methyl groups of dietary deuteriomethionine appear in the choline and creatine of the tissues.

Borsook and Dubnoff[39] in the year 1947 found that the only tissue homogenate capable of synthesizing creatine from L-methionine and glycocyamine was guinea pig liver homogenate. Furthermore the homogenate had to be fortified with adenosinetriphosphate (ATP). Borsook and Dubnoff analyzed the creatine by adsorbing the sample first on Lloyd's reagent (hydrous aluminum silicate) and then assaying the sample by the alkaline-picrate method. The rate of transmethylation was found to be, on the average, double that in rat liver slices.

In the years 1948-1949 several other in-vitro reactions demonstrating creatine synthesis, were reported. Sourkes[40] used autolyzed beef liver preparation as a source of enzyme in the preparation of creatine from methionine and glycocyamine. Fidanza[41] confirmed the finding that animal liver could catalyze the reaction between glycocyamine and methionine. He also found that the methyl group of xanthine could be utilized for creatine synthesis. However, the

utilization of xanthine was inferior to that of methionine. In other experiments Fidanza[42] demonstrated that creatine was synthesized by the crystalline lens of ox eye. The rate of this reaction approximated that catalyzed by liver preparations. Fidanza also noted that transmethylation occurred under anaerobic conditions. Barrenscheen and Valyi-Nagy[43] repeated their former experiments[30] with rat liver and muscle tissue and found that methionine sulfoxide methylated glycocyamine in the presence and absence of oxygen. They also found that Vitamin C in the presence of metal ions Co^{++} and Mn^{++} activated the reaction.

Lipmann and Kaplan[44] ably summarized the important in-vitro experiments up to the year 1949. A difference in the mechanism of methylation from choline and methionine was noted. Choline yielded its active methyl group in tissue homogenates in the absence of oxygen and with oxygen inhibitors present. Lipmann and Kaplan failed to mention Dubnoff's recent experiments[13] which showed that the methylation of homocystine from choline is small or negligible anaerobically, but that the methylation from betaine is relatively independent of oxygen tension. Methionine, on the other hand, transferred its labile methyl group to glycocyamine only in the presence of oxygen. Furthermore adenosine triphosphate (ATP) enhances the methyl transfer. Lipmann and Kaplan postulated the necessity of a phosphorylated intermediate, which most likely would be a sulphonium derivative. Cantoni[45] in 1951 confirmed this latter view when he demonstrated the biosynthesis of N^1-methylnicotinamide from methionine and nicotinamide.

Du Vigneaud[46] was able to prove that the methyl group of methionine was transferred in toto in the biosynthesis of creatine and choline. $L\text{-}C^{14}D_3SCH_2CH(NH_2)COOH$ was fed to rats for four days. Choline and creatine were isolated from the carcass. The ratio of D : C^{14} in the isolated choline and creatine was found to be the same as in the methyl group of the dietary methionine.

In the search for a "methyl carrier" Binkley and Watson[47] were able to demonstrate creatine formation in rat liver from methyl phosphate and glycocyamine. Unfortunately, methyl phosphate would not substitute for choline in the growth of rats receiving homocystine as the sulfur of their diet.

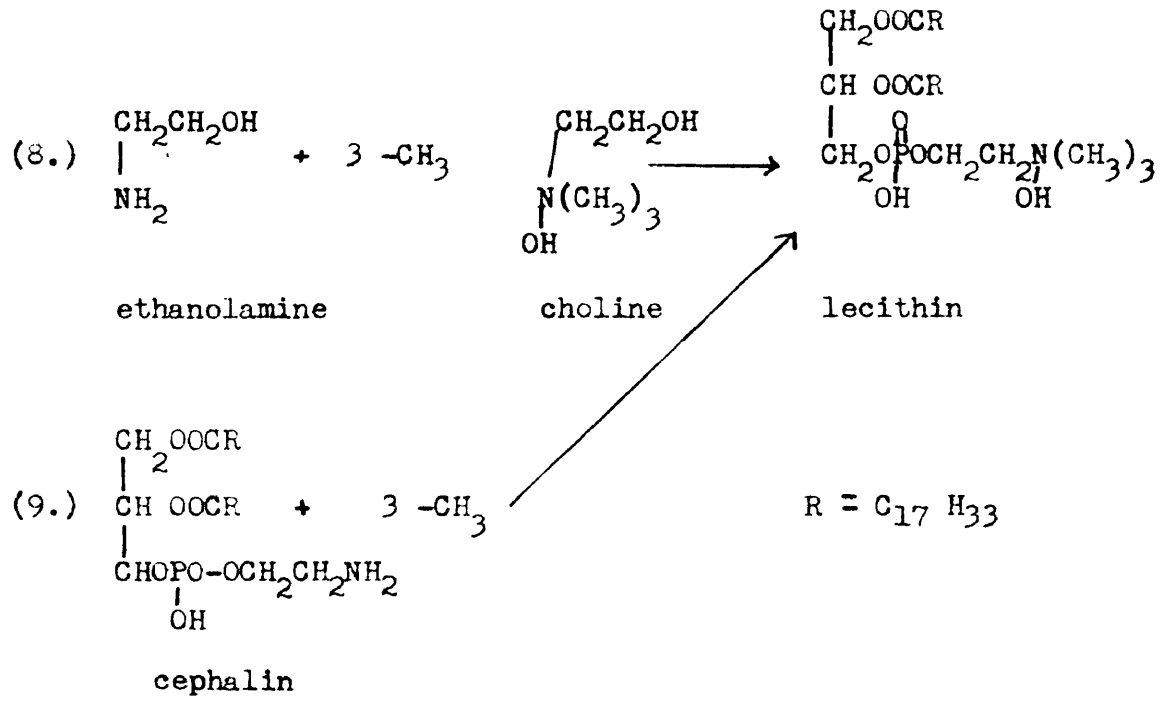
Cohen[48] in the year 1950 and Umbreit[49] in 1951 demonstrated that the formation and subsequent presence of α -keto-δ -methiolbutyric acid, due to the action of amino-oxidase, interferred markedly in the alkaline-picrate determination of creatine. Creatine "synthesized" by homogenates of beef liver from guanidoacetic acid (glycocyamine) and DL-methionine was found to be almost entirely an artifact due to the formation of the keto analogue of methionine.

Biosynthesis of Choline. By feeding rats ethanolamine labeled with isotopic nitrogen, Stetten[50] demonstrated that ethanolamine was a precursor for the biological synthesis of choline. Stetten also concluded that glycine could be reduced by the animal organism to ethanolamine. Du Vigneaud and co-workers[51] demonstrated that N^{15}-labeled choline could be isolated from rat carcass after the animal had been fed ethanolamine, labeled with N^{15}. Deuteriocholine was also isolated after the rats had been on a diet of deuteriomethionine. All the deuterium in the isolated choline was shown to be in

the methyl groups by the oxidation of choline by $KMnO_4$ to trimethylamine, which as the chloroplatinate derivative was analyzed for deuterium. Jukes[52] reported that growth of chicks on a choline-deficient diet was supported by methionine and methylethanolamine or dimethylethanolamine. Growth was not stimulated by ethanolamine and methionine. Jukes concluded that methylation of the primary methyl group in the biosynthesis of choline cannot be accomplished by the chick.

Du Vigneaud and co-workers[53] fed rats with betaine, labeled with deuterium in the methyl groups and N^{15}. Isotopic analysis of choline and creatine isolated from rat tissue showed betaine to be an extremely effective methyl donor. Since the isolated choline did not contain any isotopic nitrogen, it was concluded that the betaine molecule was not converted as a whole to choline.

Artom and Cornatzer[54] demonstrated the biosynthesis of phopholipides in animals from either ethanolamine, monomethylethanolamine, dimethylethanolamine, or choline. The animals were given a single dose of one of the compounds by stomach tube and were then injected with isotopic phosphate. The radioactivity of isolated lipides from the liver and small intestine proved that the substances tested had stimulated the phospholipide formation. In later experiments Artom and co-workers[55] observed a marked inhibition in the formation of choline-containing phospholipids in rats on a diet containing diethanolamine. Artom proposed two pathways in the formation of lecithin:



Artom reasoned that both processes would be impaired if unnatural diethanolamine were available to the animal.

Steensholt[56,57] attempted to demonstrate by in-vitro experiments the biosynthesis of choline in rat liver or muscle. Methionine was incubated with ethanolamine and animal tissue. The formation of choline was shown by the choline analysis developed by Marenzi and Cardini[58]. In later experiments Steensholt[59] demonstrated the transfer of methyl groups from methionine to ethanolamine by the disappearance of methionine, determined by the McCarthy-Sullivan method[12]. For his latest contribution to the field of trans-methylation Steensholt[60] reported that D-methionine was a considerably more efficient methyl donor to ethanolamine and dimethylethanolamine than the L isomer.

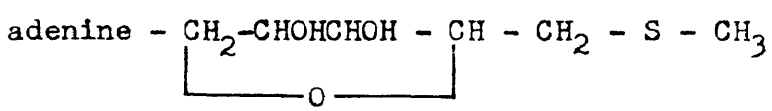
Borsook and Dubnoff[61] in their work on dimethylthetin $(CH_3)_2SCH_2COOH$ as methyl donor came to the conclusion that "methionine, for example,

does not remethylate dimethylethanolamine or dimethylglycine".

The only other report of in-vitro experiments on the biosynthesis of choline comes from Barrenscheen[62]. Rat liver slices, homogenates and extracts were shown to be active in converting ethanolamine and serine to choline by the methyl transfer of methionine. The synthesized choline was determined by the gravimetric method of Kampfhammer and Bischoff[63] by the precipitation of choline as the reineckate. No attempt was reported by Barrenscheen to identify the reineckate precipitate.

Biosynthesis of Other Compounds by Transmethylation. Perlzweig and co-workers[6] were able to show the biosynthesis of N^1-methylnicotinamide from nicotinamide in rat liver slices. Cinsa[64] demonstrated that the yield of trigonelline was not increased by incubating rabbit liver with N^1-methylnicotinic acid, but the yield was increased by the addition of choline chloride. The conclusion was that choline and not N-methylnicotinic acid transferred its methyl group. Cantoni[7,45] prepared from rat liver a cell-free enzyme extract which catalyzed the formation of N^1-methylnicotinamide in the presence of Mg^{++} and ATP. The enzyme was specific for L-methionine and nicotinamide; the enzyme was not able to methylate nicotinic acid. Sourkes[65] has written the most current summary of all transmethylation reactions studied.

The Origin and Fate of the Methyl Group. F. Lipmann noted in 1941[66] that an adenine-methio-pentose most probably of the following constitution:[67]



had been isolated from yeast. With adenosine in the prosthetic group of various enzymes, this compound might very neatly fit into the place of a methyl carrier. No further development in this respect has been reported in the literature. As already noted previously, Binkley and Watson[47] could demonstrate creatine synthesis with methyl phosphate as a methyl donor. The inference from this work might be that methyl phosphate could act as a universal methyl donor.

Several recent experiments have indicated that formate or formaldehyde might be converted to the methyl group in the animal body.[68,69,70,71] These experiments are in contrast to the older view that the animal body cannot synthesize methyl groups.

MacKenzie and co-workers[72] obtained evidence for the oxidation of the methyl group of methionine to carbondioxide in the animal organism. Animals were given by means of a stomach tube L-methionine labeled with C^{14} in the methyl group. The CO_2 which was exhaled by the animal was collected in $Ba(OH)_2$ over a period of twelve hours. 17% of the labile methionine methyl group showed up in the CO_2 as measured by the radioactivity of the $BaCO_3$. After the animal was sacrificed, greatest radioactivity was found in kidney, liver, and the adrenal gland. Roth and Allison[73] also postulated that the methyl group of methionine might be oxidized to CO_2.

Oxidation of Methionine. Krebs[74] has written an up-to-date review article on the oxidation of amino acids. The general equation for the oxidation of α - amino acids is:

(10.) $$RCH(NH_2)COOH + \tfrac{1}{2}O_2 \longrightarrow RCOCOOH + NH_3$$

The enzyme catalyzing the oxidation of D-amino acids has been isolated and identified as a dinucleotide. The enzyme has been found to be much more concentrated in rat kidney than in rat liver. The guinea pig exhibits only moderate enzyme activity in both liver and kidney. The method used most widely for the measurement of enxyme activity is the manometric determination of oxygen uptake in the presence of D-alanine, the most active amino acid.

The L-amino acid oxidase is particularly unstable in the impure form, for example in crude tissue preparations. Since in this study only crude tissue preparations were used, this historical review will be limited to D-amino acid oxidase.

Knoop[75] observed in 1910 that the first phase of the metabolism of amino acids was oxidative deamination of one stereoisomer in preference to the other. Krebs[76,77] in the year 1932 showed that deamination of D-amino acids took place to the greatest extent in the kidney. Krebs established that for each molecule of oxygen two molecules of α-keto acid were formed. He isolated the α-keto acid as the 2,4 dinitrophenylhydrazone. The quantitative determination of the keto acid was done manometrically by its enzymatic decarboxylation with yeast extract. The following amino acids showed activity: alanine, valine, leucine, norleucine, phenylalanine, and serine. Glycine showed no activity. DL-methionine was shown to have a high rate of oxidation by acetone-dried kidney powder[78]. The oxygen uptake was measured by the Warburg technique. As an example, 0.5 ml of 0.33 M DL-methionine solution showed an uptake of 139 microliter oxygen after ten minutes and 269 microliter oxygen after twenty minutes.

Bernheim and Bernheim[79] were able to show with a purified kidney preparation that two molecules of DL-methionine were oxidized by one atom of oxygen. In 1936 Bernheim and Gillaspie[80] identified the α-keto acid derived from the oxidation of methionine by rat kidney and liver slices as α-keto-γ-methiolbutyric acid. They isolated this α-keto acid as the phenylhydrazone and determined its molecular weight iodimetrically. The iodimetric titration also served as the basis for the quantitative analysis of α-keto acids. In the case of α-keto-γ-methiolbutyric acid only a yield of 66%, based on theory, was obtained.

An alternate method for the estimation of α-keto acids by titrating the bisulfite addition compound with a standard solution of iodine, proved impossible in the case of α-keto-γ-methiolbutyric acid.

Bernheim and Gillaspie also demonstrated the formation of H_2O_2 during the oxidation of amino acids, by the conversion of hemoglobin to methemoglobin which could be observed visually by the disappearance of the red color due to hemoglobin.

Klein and Handler[82] demonstrated that D-methionine was oxidized 97% by hog kidney extract. This figure is based on the initial amount of unnatural isomer of methionine present and the amount of oxygen which was absorbed.

Waelsch and Borek[83] prepared α-keto-γ-methiolbutyric acid by incubating methionine and kidney slices. The keto acid was characterized as the 2,4 dinitrophenylhydrazone.

Waelsch[84] observed that when rats were fed large amounts of methionine, they would excrete the corresponding keto acid in their urine. This observation confirmed previous work that the kidney

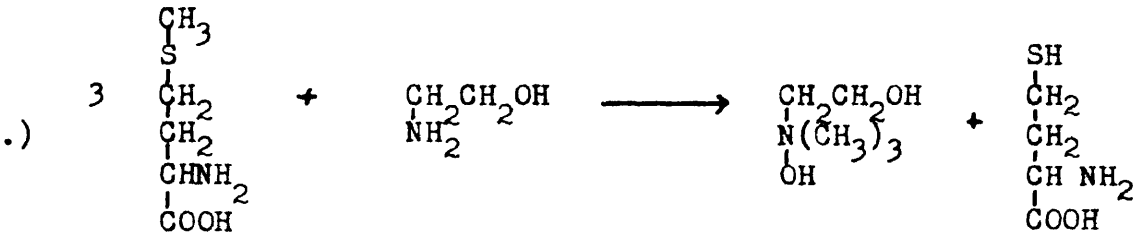
is the locale for the deamination of amino acids.

Kayhill and Rudolph[85] synthesized the barium and sodium salt of α-keto-γ-methiolbutyric acid. They then fed the sodium salt to rats and observed that the keto analogue of methionine was capable of replacing DL-methionine in the diet of these animals.

DISCUSSION

This study on the biological synthesis of choline was undertaken in the hope of showing that the reaction actually took place as postulated by Stetten[50] and du Vigneaud[51]:

(11.)



methionine + ethanolamine ⟶ choline + homocysteine

Only two research workers, Steensholt[56,57,59] and Barrenscheen[62] had attempted to isolate this reaction and had reported formation of choline from rat muscle and rat liver.

It was deemed most convenient to study this reaction in-vitro from three angles, the analysis of methionine, the analysis of choline, and the measurement of any gas exchange taking place. The colorimetric method for the analysis of methionine as devised by McCarthy and Sullivan[12] was selected as the best available. All samples were qualitatively analyzed for choline by the addition of ammonium reineckate solution with the subsequent precipitation of choline reineckate. Oxygen uptake and CO_2 production were measured in a Warburg respirometer. These analytical procedures are discussed in detail in the experimental section of this thesis.

Liver and muscle of several species of animals were tested first for degree of enzymatic activity when incubated with

methionine and ethanolamine. Experimental Runs 1, 2, and 3 in which rabbit tissue was used, showed very little or no oxygen being taken up. Since no blank was used for Run 2, the "oxygen absorbed" was probably due to endogenous respiration. In nine out of ten samples no methionine disappeared. Guinea pig liver homogenate (Experimental Run 4) gave similar results. One sample indicated a slight uptake of oxygen. Addition of adenosine triphosphate (ATP) and Vitamin B_{12} to various samples did not initiate any reaction. The rabbit and guinea pig were thus found to be unsuitable for this work.

Fresh rat liver slices and homogenates showed good activity in all experimental runs. Oxygen uptake was recorded in those experiments in which methionine was one of the substrates. Methionine also disappeared in the majority of these experiments. It was, therefore, decided to use rat liver slices and homogenate throughout this work.

In order to proceed systematically in this study, it was necessary to repeat and evaluate the work of Steensholt and Barrenscheen.

Steensholt's Experiments.[56,57,59,60] Steensholt incubated either minced rat muscle tissue or minced rat liver tissue with ethanolamine in the absence and presence of methionine. At the end of the incubation period, the samples were analyzed for choline by the method of Marenzi and Cardini[58]. In all of his reported experiments Steensholt was able to show that the photometer readings of the samples containing methionine were greater than the readings of the samples which did not contain methionine. No quantitative results were given, and the work was thus at best only qualitative.

Steensholt concluded from his results that choline must have been formed in the muscle and liver of his rats. Throughout his work Steensholt used McIlvaine's buffer with an approximate pH of 7.2 for his physiological and necessarily isotonic medium.

Two years later Steensholt wrote:[59]

> The present writer also .... occupied himself with the in-vitro synthesis of choline from ethanolamine .... with methionine as methyl donator in a suspension of tissue pulp. In this work the increase in the amount of choline .... in the reaction mixture was determined and used as a measure of the rate of reaction of the metylation process. However .... this method suffers from the defect that ethanolamine interferes to some extent with the determination of choline .... Moreover, the action of the enzyme choline oxidase in many cases tends to decrease the amount of choline synthesized ....

For the above reasons Steensholt analyzed his samples for methionine by the method of McCarthy and Sullivan.[12] A decrease in methionine concentration was then taken as evidence of transmethylation. Steensholt made no attempt to correlate the amount of methionine disappearing with the amount of choline formed.

In his latest work on transmethylation[60] Steensholt reported that "d-methionine" was a considerably more efficient methyl donator to ethanolamine than was its stereoisomer. d-methionine must be identical to d(+) methionine, which is the unnatural form of the amino acid. Steensholt also erroneously stated that Handler and Bernheim[35] had found that ... "d-methionine is about 50% more active than l-methionine ..." in the methylation of guanido acetic acid to creatine. What Handler and Bernheim actually reported was that D-methionine is about 50% as active as the natural amino acid in the in-vitro creatine synthesis.

Attempted Repeat of Steensholt's Work. In more than twenty-five experimental runs in which conditions were kept as close to the

experimental procedure of Steensholt as possible, no formation of choline could be demonstrated by the reineckate method of Marenzi and Cardini. In no experiment did any precipitate appear after the addition of saturated ammonium reineckate to any sample.

The method for choline analysis by Marenzi and Cardini actually is the analysis of the chromium content of choline reineckate. Since ammonium reineckate also contains chromium, it is conceivable that if the washing of choline reineckate precipitate is not done with extreme care, the results of "choline formation" may be quite misleading.

Another criticism of Steensholt's work is that no mention is made of any precautions to maintain a constant pH throughout a particular experimental run. For example Steensholt gave a description of the contents of an experimental flask as follows:

> . . . . In a small flask A were placed
> 0.3 g minced muscle tissue
> 0.05 ml ethanol amine
> 40 mg methione
> 4 ml McIlvaine's phosphate-citrate buffer (pH about 7.1)

McIlvaine's buffer[86] was made up from 0.1 M citric acid and 0.2 M Na_2HPO_4. By adding 0.05 ml ethanolamine to 4.0 ml of McIlvaine's buffer (pH about 7.2), the pH of the solution was observed to rise above 10. Steensholt reported the results of experiments in which the pH had been varied by different buffer compositions. If, however, he had made no provisions to keep the hydrogen ion concentration constant after the addition of unneutralized ethanolamine, Steensholt's experimental results must be interpreted with caution.

The behavior of methionine in the presence of straight ethanolamine and ethanolamine neutralized with dilute acid is compared

in Exprimental Runs Nos. 5 and 6. A much smaller amount of methionine had disappeared in the flask in which methionine and unneutralized ethanolamine were incubated with liver homogenate. Most enzyme activity is destroyed in very basic medium; this fact was brought out by these experiments.

No choline reineckate precipitated when ammonium reineckate was added to each sample. The addition of 2,4 dinitrophenylhydrazine reagent caused an immediate, yellow precipitate in those samples whose pH had been kept constant at 7.2. It was shown in later work that the yellow phenylhydrazone was the derivative of α-keto-γ-methiolbutyric acid, the keto analogue of methionine. Another qualitative test for α-keto acids proved negative in those samples where the pH exceeded 10 due to the addition of unneutralized ethanolamine. It was, therefore, demonstrated that no reaction had taken place when methionine and unneutralized ethanolamine had been incubated with fresh rat liver slices. Since Steensholt did report a disappearance of methionine in his experiments, it is conceivable that he neutralized ethanolamine, although this fact is not recorded in any of his work.

In Run No. 7 a small amount of the 2,4 dinitrophenylhydrazone was isolated. A comparison of the melting point and percentage nitrogen analyzed with the literature values[83] confirmed that the original compound was α-keto-γ-methiolbutyric acid.

From the results of Runs Nos. 8 and 10 it was concluded that the reaction involving methionine was an enzymatic reaction, dependent on the presence of atmospheric oxygen. Run No. 9

demonstrated that hog kidney powder was very active in the reaction causing a considerable disappearance of methionine, however, Run No. 29 demonstrated that rat liver was incapable of causing the disappearance of L-methionine.

The above results confirmed the belief that methionine had been acted upon by the enzyme d-amino acid dehydrogenase. The enzyme is only active under aerobic conditions and is inactivated at a pH greater than 10. This enzyme had been shown to exhibit greater activity in rat kidney than in rat liver[87] and almost no activity in the liver of the rabbit and guinea pig. These facts are confirmed by Experimental Runs Nos. 1 through 4 and No. 9.

It now remained to prove that the formation of α-keto-γ-methiolbutyric acid would be indistringuishable from the actual disappearance of methionine when applying the McCarthy-Sullivan method for the determination of methionine. Pure samples of sodium α-keto-γ-methiol butyrate were analyzed for methionine by the McCarthy-Sullivan method. The results and the evaluation of the extinction coefficient are reported in the section on "Analytical Procedures" of this thesis. The results confirmed that α-keto-γ-methiolbutyric acid produced a less intense color than that of an equivalent amount of methionine. It had thus been shown that the formation of the keto analogue of methionine would in effect show up as "loss of initial methionine". To conclude, therefore, that the methyl group of methionine had been transferred on the basis of the observation of disappearance of methionine, would be fallacious. The claim by Steensholt that D-methionine was more active in transmethylation than L-methionine, may now

be viewed in the light that D-amino acid dehydrogenase in rat liver is much more active than L-amino acid dehydrogenase.

No apparent influence on the reaction could be detected when adenosine triphosphate (ATP), Vitamin B_{12}, cytochrome-C, or diphosphonuecleotide (DPN) were added to the samples in varying concentrations.

Barrenscheen's Work and Its Evaluation. Barrenscheen[62] also claimed choline synthesis by slices, homogenates, and extracts of the liver of rats and guinea pigs. The claim was made that choline synthesis occurred using ethanolamine or serine as methyl acceptors. Barrenscheen reported that in more than thirty experiments with liver positive results were obtained. However, that due to unknown reasons, about 20% of his experiments were unsuccessful in the biosynthesis of choline.

Barrenscheen determined choline by the method of Kapfhammer and Bischoff[63]. The procedure called for a washing of the deproteinized samples with ether and absolute alcohol. The samples were then evaporated to dryness, dissolved in a minimum amount of water, and an equal volume of saturated ammonium reineckate was added to precipitate choline. After careful washing and drying choline reineckate was weighed and then dissolved in acetone. The empty gooch crucible was weighed and the calculations were made on "acetone-soluble choline reineckate". No attempts were reported to identify the "acetone soluble reineckate".

Experimental Run No. 23 was made under conditions identical to those of Barrenscheen. Dl-methionine and ethanolamine were incubated with rat liver homogenate. Barrenscheen's directions for

the isolation of choline as the reineckate were followed exactly. A pink precipitate, similar to choline reineckate in appearance, was obtained. Analysis, however, showed that the precipitate was ethanolamine reineckate. Ethanolamine reineckate was also found to be acetone soluble. The formation of an "acetone soluble reineckate" may, therefore, not be taken as conclusive evidence for choline formation.

Cohen[48] and Umbreit[49] had found that the formation of α-keto-γ-methiolbutyric acid from methionine in rat liver had caused erroneous results and conclusions as to creatine formation in-vitro. The same interference of this keto acid also makes the work on the in-vitro choline formation rather doubtful.

J. Dubnoff[88] wrote in reviewing the recent work of the biosynthesis of choline:

> We have never been able to repeat any of the work of Steensholt and Barrenscheen on any phase of transmethylation..... these workers have claimed activity in muscle of several reactions which we have been unable to repeat. The results are usually just beyond the experimental error and of doubtful significance. We have even used tracers without success.....

Evidence for Choline Synthesis by the Use of Choline Oxidase. It has been discovered recently that Co^{++} when added to rat liver homogenate inhibits the activity of choline oxidase about 84%.[89] The oxidation of choline by rat liver had been observed earlier by Mann and Quastel[90], and the oxidation product had been identified as betaine aldehyde. The reaction can thus be written:

(12.) $$(CH_3)_3\underset{\underset{OH}{|}}{N}CH_2CH_2OH + \tfrac{1}{2}O_2 \longrightarrow (CH_3)_3\underset{\underset{OH}{|}}{N}CH_2CHO + H_2O$$

In preliminary experiments choline was incubated with rat liver homogenate in the absence and presence of Co^{++} ions. The measured oxygen uptake in those samples containing Co^{++} was about 30% smaller than the oxygen uptake by samples containing no Co^{++} (Experimental Run No. 15). Results from similar experiments, Runs Nos. 16 and 18, were erratic, and no definite conclusions as to the effectiveness of Co^{++} ions inhibiting choline oxidase activity, were drawn.

The results of Experimental Run No. 17 presented evidence for the formation of choline from ethanolamine and methionine. Three samples containing methionine and ethanolamine were incubated with fresh rat liver slices. Choline oxidase activity had been demonstrated only in rat liver homogenate and not slices[89], and thus any choline which might be formed in the course of these experiments should not be oxidized. After a six-hour incubation period, aliquots of these samples were then incubated with fresh rat liver homogenate. $CoCl_2$ was added to one series of flasks. The results showed about 75% inhibition of oxygen uptake in this series compared to a similar series of flasks containing no Co^{++}.

The results of Run No. 18 showed a 39% inhibition in oxygen uptake of those samples which contained methionine, ethanolamine, and rat liver homogenate plus Co^{++} ions. However, the same homogenate produced no such inhibition with choline in the presence of Co^{++} ions. The significance of these results were, therefore, rather doubtful, because Co^{++} ions failed to inhibit the oxidation of known choline.

In the case of Run No. 18-A ethanolamine was substituted by acetyldimethylethanolamine as methyl acceptor. If transmethylation occurred, acetylcholine would result which in the absence of any choline esterase poisons would be easily hydrolyzed to choline by rat liver homogenate.

(13.) $(CH_3)_2NCH_2CH_2OCOCH_3 + {}^{-}CH_3 + (OH)^{-} \longrightarrow (CH_3)_3\overset{OH}{\overset{|}{N}}CH_2\ CH_2OCOCH_3 \xrightarrow{H_2O} (CH_3)_3\overset{OH}{\overset{|}{N}}CH_2CH_2OH + CH_3COOH$

The influence of Co^{++} ions on the rate of oxygen uptake should also be observed in this run if transmethylation actually took place. Sample No. 1, Run 18-A, containing Co^{++} ions exhibited a 66% decrease in oxygen uptake compared with Sample No. 2 which contained no Co^{++}. The two samples which contained only methionine showed almost no inhibition with Co^{++}. Choline analysis of all samples at the end of the incubation with ammonium reineckate proved negative. It was expected that those samples which had contained Co^{++} ions, acetyldimethylethanolamine, and methionine would give a positive choline test, since the oxidation of choline formed should have been inhibited.

Results of Experimental Run No. 23 were completely negative. The observed oxygen uptake of six samples, three of which contained Co^{++} ions, and all of which contained methionine and ethanolamine, was the same within limits of the Warburg technique.

The above results cannot be offered as conclusive evidence for

choline formation in-vitro by rat liver.

Oxidation of Methyl Group of Methionine. Mackenzie[72] had obtained evidence by the use of $C^{14}H_3SCH_2CH_2CH(NH_2)COOH$, given to animals by stomach tube, that $C^{14}O_2$ was produced by the animal. He concluded that some of the labile methyl group of methionine was being oxidized to CO_2.

By the use of the "Direct Method" for carbon dioxide[91] the results of Experimental Run No. 19 indicate the formation of CO_2 by incubating samples of methionine with rat liver slices. The ratio of O_2 / CO_2, taking the average values of four samples, was calculated to be 1.85. The following equation might be proposed:

$$\underset{\text{methionine}}{CH_3SCH_2CH_2CH(NH_2)COOH} + 1\tfrac{1}{2}O_2 \longrightarrow \underset{\text{homocysteine}}{SHCH_2CH_2CH(NH_2)COOH} + CO_2 + H_2O$$

From this postulated equation the ratio $\frac{\text{oxygen absorbed}}{CO_2 \text{ given off}}$ calculates to be 1.5.

Experimental Run No. 20 in which methionine plus ethanolamine were incubated with rat liver slices, demonstrated that no carbon dioxide was formed. Evidence is thereby obtained that the labile methyl group of methionine is oxidized by rat liver, but that the methyl group is protected in the presence of ethanolamine.

Biosynthesis of Homocysteine. In all previous work on transmethylation involving methionine, it had been postulated that homocysteine was formed after methionine was demethylated. Nobody has ever been able to demonstrate the presence of free homocysteine in the animal body.

In experimental Runs Nos. 13 and 17 it was proposed that even

if homocysteine could not be isolated as such, the presence of a free sulfhydryl group might be detected by well known methods. Method A, involving the use of phosphotungstic acid reagent, and Method B, involving iodine titration, are both described in detail in the EXPERIMENTAL part of this thesis. In Run No. 13 DL-methionine, in the presence and absence of ethanolamine and glycocyamine, was incubated with rat liver homogenate. By methionine analysis it was ascertained that appreciable amounts of methionine had disappeared after the incubation period. Analysis for free sulfhydryl groups of all samples by Method A proved negative. In Experiment No. 17 methionine and ethanolamine were incubated with rat liver slices, and all samples without analyzing first for methionine, were analyzed for free sulfhydryl group by Method B. Again this test proved negative.

It was concluded, therefore, that in the case of methionine and ethanolamine, no homocysteine had been formed, although methionine had been shown to disappear. Since transmethylation involving ethanolamine had not been established, it could only be said that methionine disappeared by another mode than transmethylation. However, in the case of methionine and glycocyamine, where transmethylation had been repeatedly demonstrated by others to occur in-vitro,[24] no homocysteine was produced. It should also be noted that the ratio "methionine:O_2" for the case of methionine and methionine plus ethanolamine was found to be close to 2, whereas in the case of methionine and glycocyamine this ratio was calculated to be 1.27. The significance of this ratio is discussed more fully in a later section.

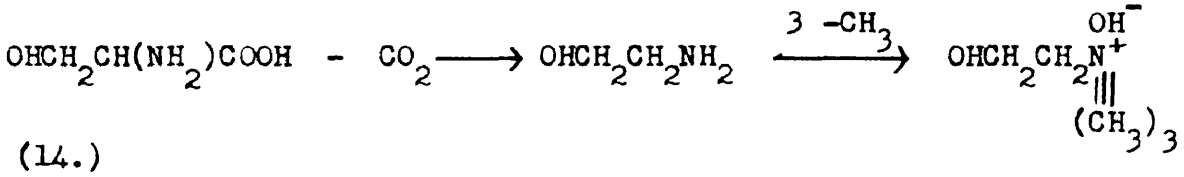
Methyl Phosphate as Methyl Donor. Binkley and Watson[47] have

demonstrated the in-vitro formation of creatine from glycocyamine and methyl phosphate by rat liver. In Experimental Runs Nos. 21 and 22 monomethyl disodium phospate was incubated with ethanolamine and rat liver homogenate. In Run No. 21 an attempt was made to demonstrate the formation of choline by the use of homogenate with and without Co^{++} ions. If choline was being formed, those samples containing Co^{++} should take up oxygen at a slower rate than those samples without cobalt. Experimentally it was found that practically no reaction, involving oxygen, took place with and without Co^{++} ions. In Run No. 22 the formation of choline from methyl phosphate and ethanolamine was to be demonstrated by the use of ammonium reineckate to precipitate any formed choline. No such precipitate appeared. From these experiments it was concluded that methyl phosphate was unable to serve as methyl donor in the formation of choline from ethanolamine.

Acetyldimethylethanolamine as Methyl Acceptor. The methylation of acetyldimethylethanolamine would result in the formation of acetylcholine. If an experiment were devised in which the action of choline esterase, which causes the hydrolysis of acetylcholine, could be inhibited, acetylcholine might be isolated. The synthesis of acetylcholine would also prevent the action of choline oxidase, the enzyme which catalyzes the oxidation of choline. Eserine is known to be a strong inhibitor of choline esterase[92]. Experimental Run No. 24 was devised in such a way as to test the validity of the above postulation. Methionine, acetyladimethylethanolamine and eserine were incubated with rat liver homogenate. At the end of the incubation period the samples were tested for acetylcholine

by the reineckate precipitation method. (It was previously ascertained that acetylcholine would also precipitate as reineckate under the same conditions as choline.) An immediate precipitate formed in all samples, and was identified as the reineckate of acetyldimethylethanolamine, the starting material. This experiment, therefore, proved unsuccessful.

DL-serine as Methyl Acceptor. Stetten[50] and Arnstein[71] have found evidence by isotopic experiments that the β carbon of serine was incorporated into the choline molecule in the biosynthesis of choline by the rat. The reaction was postulated as follows:



(14.)

serine ethanolamine choline

In Experimental Runs Nos. 25, 27, 28, and 29 DL-methionine, L-methionine, and DL-serine were incubated with rat liver homogenate. With the exception of one sample, no disappearance of methionine could be detected in any sample. All samples were analyzed for free choline by the addition of ammonium reineckate, and no precipitate indicating choline formation, was observed. The uptake of oxygen in Run No. 27 may be attributed to the oxidation of methionine and/or serine. These experiments showed that no choline was produced by the transfer of methyl groups from methionine to serine or ethanolamine, which would be formed after serine was decarboxylated.

Biosynthesis of Lecithin. Artom[54,55] had obtained evidence for the biosynthesis of phopholipids from ethanolamine and choline. If choline was formed by transmethylation, would it be immediately incorporated as phopholipid? In order to test this postulation, Run No. 28 was set up so that samples at the end of the incubation period were analyzed for "free choline" and "tied-up choline". Preliminary experiments, which are discussed in detail in the EXPERIMENTAL part, demonstrated that rat liver contained no detectable amounts of free choline, when dealing with about one gram of tissue. However, after a twenty-four hour acid hydrolysis of the tissue with HCl, a reineckate precipitate was obtained and identified as choline reineckate. Steensholt[56] and Barrenscheen[62], on the other hand, had reported the presence of "free choline" in their blanks, which contained only animal tissue and ethanolamine.

Run No. 28 provided conclusive evidence that methionine and serine or ethanolamine had not been able to synthesize lecithin. As a matter of fact, the blank, which consisted only of rat liver homogenate, gave a slightly greater choline analysis than the other two samples.

In Run No. 29 it was hoped to investigate the possibility of the methylation of cephalin to form lecithin (Equation No. 9). When L-methionine was incubated with cephalin and rat liver homogenate, about 3% of the starting methionine disappeared. Evidence was found for keto-acid formation, indicating activity of l-amino acid dehydrogenase in this particular animal. Evidence was found that no lecithin had been formed, but that some cephalin

had been hydrolyzed to ethanolamine. No positive evidence was found for the biosynthesis of lecithin by a transmethylation reaction.

Influence of ATP on Disappearance of Methionine. Cantoni[45] had proposed a methionine-phosphate intermediate before methionine would be able to donate its methyl group for a transmethylation reaction. If the isolated system, therefore, was not capable of regenerating adenosine triphosphate, then sufficient amounts of ATP should be provided for the system. In Run No. 14 it was proposed to study the influence of equimolecular amounts of methionine and ATP in the presence of "methyl acceptors" such as ethanolamine and serine. The results showed that methionine disappeared at about the same rate as in other experiments when only small amounts of ATP or none at all had been added to rat liver homogenates. In the case of methionine and DL-serine 4.7% of the initial methionine had disappeared. This observation may be compared to Runs No. 25 and No. 28 in which no methionine disappeared. However, no choline could be detected in any of the samples of Run. No. 14, and the disappearance of methionine could only be ascribed to d-amino acid dehydrogenase. It could not be concluded, of course, that ATP had no influence on the ease of methyl transfer from methionine, because in this case no transmethylation could be demonstrated.

Determination of Ratio "Methionine Disappearing: Oxygen Uptake". Krebs[76,77] and Bernheim[79] had been able to establish the following equation for the oxidative deamination of d-amino acids:

$$RCH(NH_2)COOH + \tfrac{1}{2} O_2 \longrightarrow RCOCOOH + NH_3$$

By measuring the uptake of oxygen for a known amount of amino acid, the validity of the above equation could be ascertained. However, the reaction would have to be followed to its completion. Another method would be to measure the amount of α-keto acid formed in the course of the reaction. This latter method in the case of methionine gave very unsatisfactory results.[80]

In the course of the study on transmethylation, involving methionine, a new and very convenient method to establish the stoichiometric relationship between methionine and oxygen in the oxidation of methionine was developed.

At the end of the incubation period of samples containing methionine, with and without ethanolamine, and rat liver, each sample was analyzed for methionine by the method of McCarthy and Sullivan[12]. This analysis actually accounted for methionine and α-keto-γ-methiolbutyric acid, which had been formed. The calculations for "true" methionine content are discussed fully in the EXPERIMENTAL part of this thesis. By determining the initial methionine content, the amount of methionine which had disappeared in the course of the reaction could thus be obtained by simple substraction. Since the reaction was followed in a Warburg apparatus, the amount of oxygen which was absorbed was also determined. Theoretically it was expected that two molecules of methionine would react with one molecule of oxygen, so that the ratio of "methionine disappearing:: oxygen absorbed" would be 2. Experimentally this ratio calculated in Runs Nos. 11, 12, and 13 had an average value close to 2. This ratio did not change when

either methionine alone or methionine and ethanolamine were incubated with rat liver. This observation is further evidence that ethanolamine has no influence on the course of the deamination of methionine by rat liver. However, the ratio in the case of methionine and glycocyamine decreased to 1.3, whereas with the same homogenate the ratio of 2 was achieved in the case of methionine and methionine plus ethanolamine. The transfer of the methyl group from methionine to glycocyamine had been demonstrated several times by in-vitro experiments.[24] Therefore, may the deviation of the ratio from 2 in the case of methionine and glycocyamine be considered as additional evidence for another reaction than merely the oxidative deamination of methionine? Since Run No. 13 represented the only attempt to study the action of methionine on glycocyamine, no definite conclusions may be drawn as to the mechanism of the methyl transfer.

EXPERIMENTAL

ANALYTICAL PROCEDURES

Analysis of Methionine. A modified procedure of the method of McCarthy and Sullivan[12] was used for the analysis of methionine in this work. The procedure is as follows:

A five milliliter solution containing methionine was pipetted into a one hundred milliliter volumetric flask, fitted with a ground glass stopper. One milliliter of 14.3 N sodium hydroxide, one milliliter of a one percent aqueous solution of glycine, and 0.6 milliliter of a ten percent aqueous solution of sodium nitroprusside (freshly made up) were added with mixing after each of the above additions. The flask was then placed in a water bath at 35° to 40°C for ten minutes. The flask was then cooled in ice water for two minutes. Five milliliters of a hydrochloric-phosphoric acid mixture (nine volumes of concentrated hydrochloric acid to one volume of eighty-five percent phosphoric acid) was added, with shaking during the addition. The solution was shaken very thoroughly for one minute, cooled at room temperature for five minutes, and the volumetric flask was then made up to the mark with distilled water. A deep red color developed after the addition of the acid mixture.

The percent transmission of the colored solution was measured with a Beckman ultra-violet spectrophotometer at a wave length of 510 mu. A blank was used in order to balance the instrument at one-hundred percent transmission. For the blank five milliliters

of distilled water in place of the solution containing methionine was used, and the sample was treated in an identical way as was described above.

A standard curve plotting percent transmission against concentration of methionine was prepared. The extinction coefficient was calculated from the per cent transmission. Results are given in Tables 1 and 2 and Figure 1.

TABLE 1. Colorimetric analysis of methionine. Per cent light transmission at 510 mu.

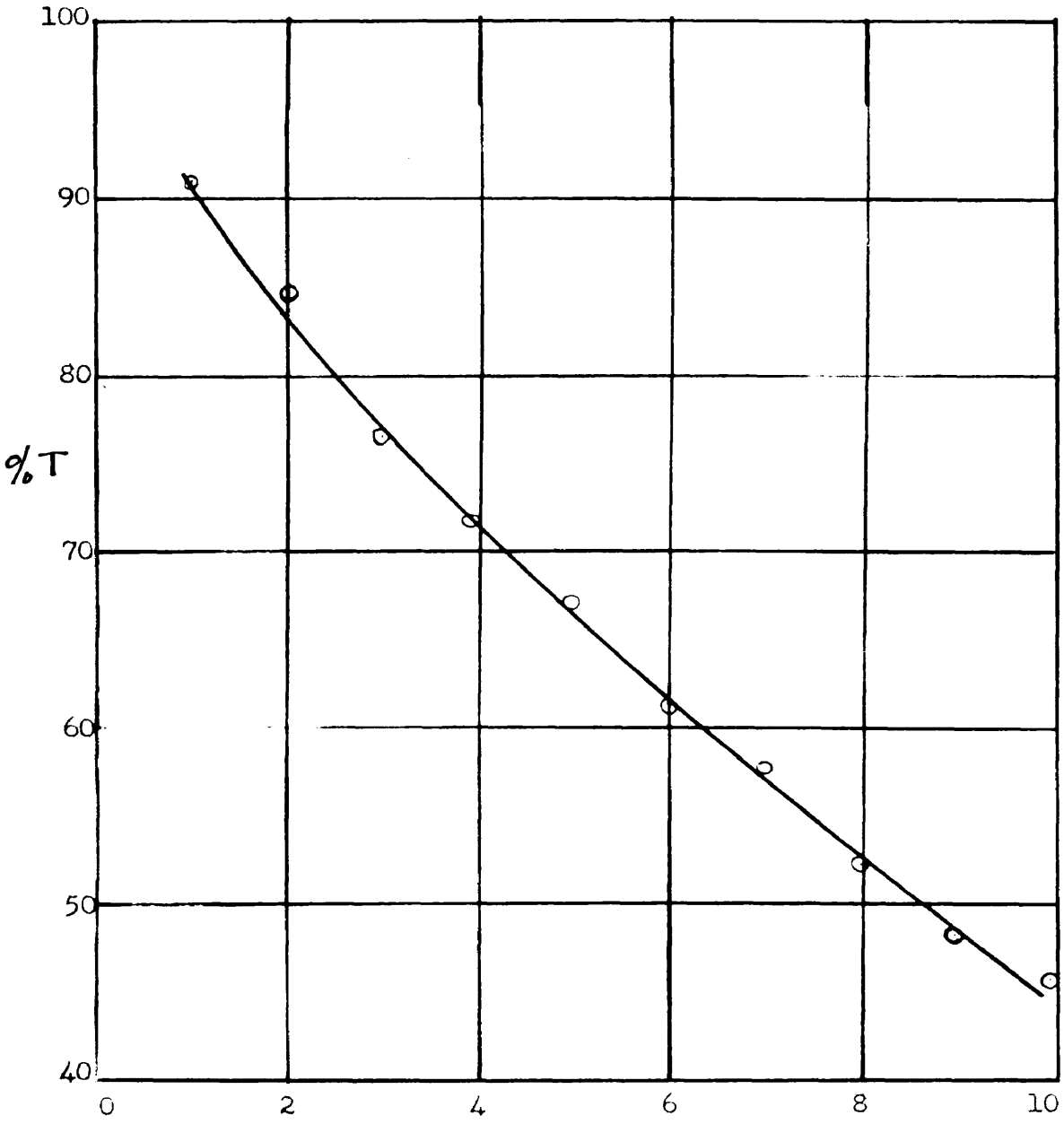
Milligrams DL methionine per 100 ml solution	per cent transmission at 510 mu.
1.0	90.8
2.0	84.5
3.0	76.5
4.0	71.5
5.0	67.1
6.0	61.2
7.0	57.7
8.0	52.2
9.0	48.3
10.0	45.8

TABLE 2. Colorimetric analysis of methionine.

Extinction coefficient at 510 mu.

milligrams DL methionine per 100 ml solution	extinction coefficient
3.0	0.039 $cm^{-1}mg^{-1}/100$ ml
4.0	0.031
5.0	0.035
6.0	0.037
7.0	0.034
8.0	0.035
9.0	0.035
10.0	0.034
AVERAGE:	0.035

FIGURE 1. Per cent transmission at 510 mμ against concentration of methionine.



Sample Calculations

The Beer-Lambert's law can be expressed as follows:

(1.) $$- \log T = kcl$$

T : per cent transmission/100

k : extinction coefficient

c : concentration expressed as mg/100 ml

l : thickness of cell (1.0 cm)

For 7.0 mg methionine, the observed T was .577. Substituting in Equation (1.), the following expression is obtained:

$$k = \frac{-\log .577}{7.0 \times 1.0} = \frac{.23882}{7.0} = 0.0341$$

Analysis of α-Keto-γ-methiolbutyric Acid. The method of analysis is identical with that used for the determination of methionine. Results are given in Table 3.

TABLE 3. Analysis of α-keto-γ-methiolbutyric acid. Per cent light transmission at 510 mu. Calculated extinction coefficient by the use of Equation (1.).

milligrams per 100 ml	per cent transmission	extinction coefficient
3.01	93.9*	
	94.5	0.009 $cm^{-1}mg^{-1}/100$ ml
6.10	90.0	
	89.0	
	90.2	
	90.2	0.008
7.32	87.9	
	87.5	
	88.0	0.008
8.54	84.5	
	84.5	
	85.0	0.009
	AVERAGE.....	0.009

Sample of sodium salt of α-keto-γ-methiolbutyric acid obtained from Dr. Alton Meister, Cancer Institute, National Institutes of Health, Bethesda, Md.

*The various values for "per cent transmission" represent different spectrophotometer readings for one particular sample.

Analysis of Methionine in the Presence of α-Keto-γ-methiolbutyric Acid. According to Weissberger[94] if two or more components of a solution impart a color to the solution, and if the colors do not interfere with each other, then the following equation may be set up:

$$(2.) \qquad -\log \frac{I}{I_o} = (k_1c_1 + k_2c_2 + \ldots) \times d$$

k = extinction coefficient

$\frac{I}{I}$ = observed transmission/100

c = concentration of each component

d = thickness of cell

If a solution contains, therefore, two components, by knowing the extinction coefficient of each component at two wave lenghts and by determining the transmission at these wave lengths, the concentration of each component may be obtained.

Due to the fact that the molecular weights of methionine (149.21) and that of α-keto-γ-methiolbutyric acid (148.17) differ by less than 1%, the following equation may be set up:

$$(3.) \qquad -\log I_o = \left[k_1(c_o - c_2) + k_2c_2\right] \times d$$

k_1 = extinction coefficient of α-keto-γ-methiolbutyric acid

k_2 = extinction coefficient of methionine

c_2 = concentration of methionine

c_o = concentration of methionine at start

The extinction coefficient at only one wave length need be known in order to solve this equation.

In order to test the validity of Equation (2.), a fifty milliliter solution, containing 1.55 mg of α-keto-γ-methiolbutyric acid and 1.55 mg of DL methionine, was analyzed for methionine by Sullivan and McCarthy's method. The observed percent transmission at a wave length of 510 mu was 73.2%.

The following values were substituted in Equation (2.)

c_1 = concentration of α-keto-γ-methiolbutyric acid

= 3.10 mg/ 100 ml solution

c_2 = concentration of methionine

= 3.10 mg/ 100 ml solution

k_1 = .00811

k_2 = .0350

d = 1.0

$$(.00811 + .0350) 3.10 = -\log \frac{I}{I_o} = -.735$$

% transmission (found) : 73.2

% transmission (calculated) : 73.5

Qualitative Tests For α -Keto Acids. Two methods were used in this work.

Method A. Addition of several milliliters of 2,4 dinitrophenylhydrazine reagent in 2N HCl and the appearance of a bright yellow precipitate was taken as evidence for formation of keto acid.

Method B. The addition of 0.6 ml of freshly prepared sodium nitroprusside to a sample which had been made basic with 1.0 ml of 14.3 N NaOH resulted in the appearance of a fleeting red color, whose intensity depended on the concentration of α -keto acid present. This test was confirmed with samples of pyruvic acid and α -keto-γ-methiolbutyric acid. It was not concluded, however, that either test was specific for α -keto acid formation. These tests were only used as additional evidence for the production of "keto" groups.

Determination of Choline. This method is based on the color developed when choline reineckate is dissolved in acetone. The method used here was described by Beattie[95] and also in Biological Symposia[96]. The method of analysis is as follows:

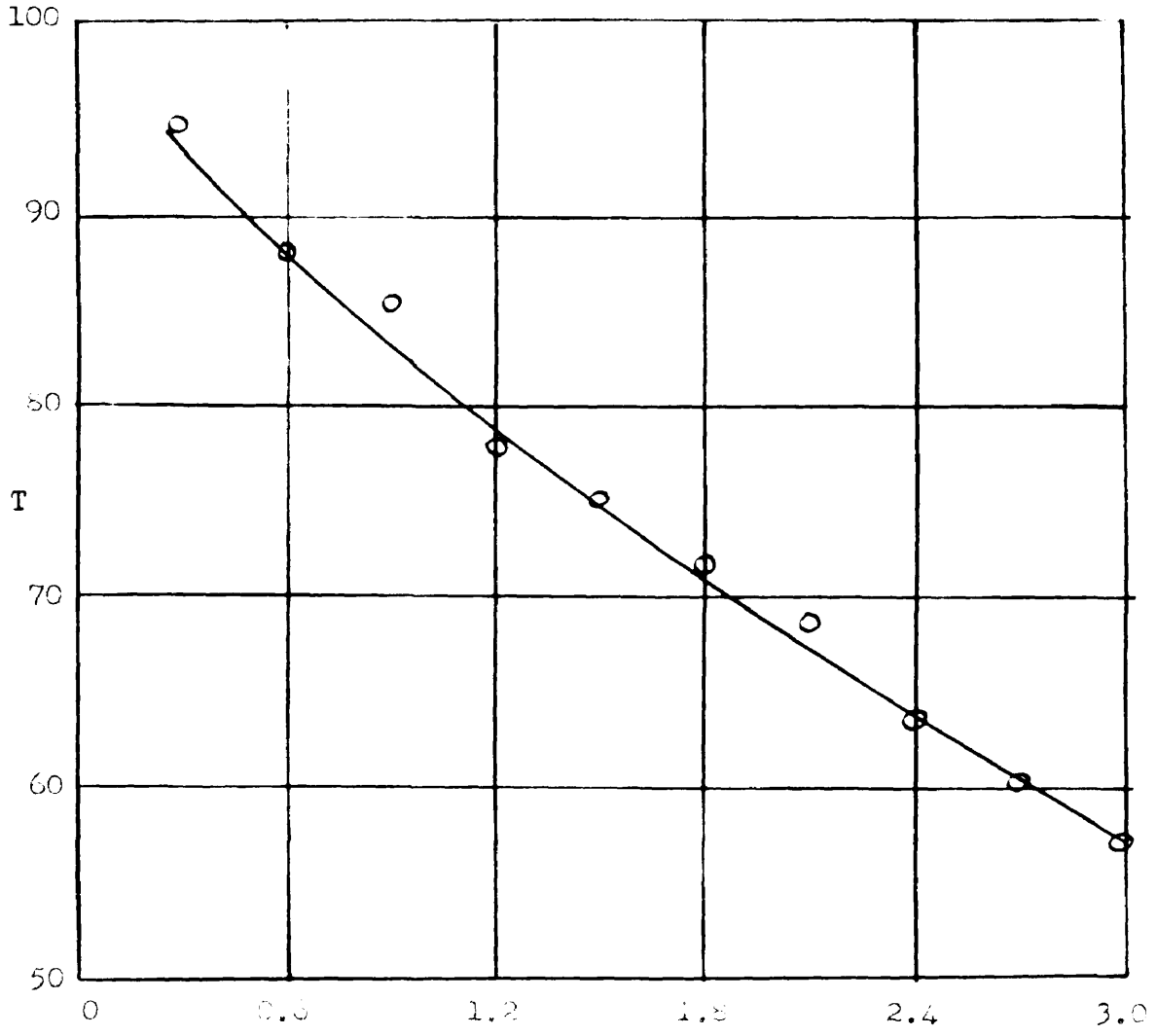
The test solution containing choline was made basic to thymoephthalein (pH 10). One-sixth volume of a two percent aqueous ammonium reineckate solution ($NH_4[Cr(CNS)_4(NH_3)_2]$) was added, and the flask was placed in the refrigerator for three hours. The choline reineckate precipitate was then poured onto a previously chilled sintered glass filter. The precipitate was washed twice with 2.5 milliliter portions of ice cold water saturated with choline reineckate and once with alcohol similarly saturated. The precipitate was then dried in air. The precipitate was then dissolved on the filter by several portions of acetone and the resultant solution gently sucked into a ten milliliter volumetric flask. The flask was made up to volume with acetone. The per cent transmission of this solution was then determined at 525 mu using acetone as a blank.

In this work a curve plotting per cent transmission at 525 mu against concentration choline was constructed. Acetone solutions of choline reineckate which contained from 0.3 to 3.0 mg choline were used. Results are given in Table 4, and Figure 2.

TABLE 4. Colorimetric determination of choline as choline reineckate. Per cent light transmission at 525 mu.

milligram choline per 10.0 milliliter acetone solution	per cent transmission
0.3	94.5
0.6	88.0
0.9	85.2
1.2	77.8
1.5	75.0
1.8	71.8
2.1	68.6
2.4	63.1
2.7	60.3
3.0	57.0

FIGURE 2. Per cent transmission at 525 mu against concentration of choline.



Determination of Free Sulfhydryl Groups. Two methods were adapted and used for this work.

A. Phosphotungstic Acid Reagent Method.

This reagent was prepared according to directions given by Snell[97]. Five milliliters of an aqueous solution containing 5.0 milligrams cysteine hydrochloride was neutralized with 0.2 N sodium hydroxide. Five milliliters of the reagent was then added to the test solution, and a deep-blue color developed. The appearance of the blue color was taken as evidence for the presence of free sulfhydryl groups

B. Iodine Titration Method.

Hess and Sullivan[98] developed a method for the determination of free sulfhydryl groups in proteins. This method is as follows:

Dilute hydrochloric acid is added to an aqueous solution, containing free sulfhydryl groups, so that the final concentration is two per cent with respect to hydrochloric acid. 2.5 milliliters of 4% hydrochloric acid and 2.5 milliliters of 5% aqueous potassium iodide are added. The solution is cooled to 20°C and titrated to a permanent yellow color with M/600 potassium iodate solution that has been standardized against cysteine hydrochloride similarly treated. The results are calculated in terms of cysteine.

0.636 grams of cysteine hydrochloride was dissolved in water to a final volume of 25.0 milliliters. The KIO_3 solution was titrated against three 5.0 milliliter aliquots of the cysteine hydrochloride solution. The results of this titration are given in Table 5.

TABLE 5. Determination of free sulfhydryl groups by titration with potassium iodate.

Sample No.	Contents	milliliters KIO_3 solution	
I	12.72 mg cysteine hydrochloride	5.110	
II	12.72 mg cysteine hydrochloride	4.990	
III	12.72 mg cysteine hydrochloride	5.019	AVERAGE: 5.040
IV	5.0 ml distilled water	0.140	

4.900 milliliters of KIO_3 solution is equivalent to 12.72 mg of cysteine hydrochloride.

PREPARATIONS

Choline Reineckate. 0.5 gram of choline hydrochloride was dissolved in 100 milliliters of water, and 100 milliliters of saturated aqueous ammonium reineckate, containing about four grams of ammonium reineckate, was added. The solution was acidified with four drops of concentrated hydrochloric acid, at which time a pink precipitate appeared. The flask was set aside in a refrigerator overnight. The precipitate was then centrifuged and the supernatant liquid decanted. The precipitate was dissolved in 100 milliliters of acetone and water added drop by drop until the solution became cloudy. The flask was again kept in a refrigerator overnight. The precipitate was centrifuged and washed with 95% alcohol, the liquid decanted and the precipitate washed with anhydrous ether. The precipitate was then filtered and dried in vacuo over concentrated sulfuric acid. The decomposition point was observed at about 254°C. No literature values on the decomposition point could be found.

Analysis:* Calculated for $C_9H_{20}ON_7CrS_4$: Nitrogen, 23.20%

Found: Nitrogen, 22.80, 23.50%

---

*All microanalyses for carbon, hydrogen, and nitrogen were carried out by Mrs. Mary Aldridge and Mr. Byron Baer.

Acetyldimethylethanolamine hydrochloride. 10.0 grams of ethylene oxide and 10.0 grams of dimethylamine were placed in a 250 milliliter round bottom flask, and the reaction mixture was kept cold for one hour. The product was distilled at 18 millimeter pressure and the fraction distilling at 89°C collected. The distillate was extracted with ether and dried over anhydrous magnesium sulfate. Five milliliters of acetylchloride was added to the dry ether extract, and a white precipitate which appeared collected on a filter. The precipitate was washed several times with dry ether and recrystallized from acetone-ether. The melting point was observed at 132-133°C. No literature values on the melting point of this compound could be found.

Analysis: Calculated for $C_6H_{14}O_2NCl$: Chlorine, 21.17%

Found: Chlorine, 21.30%

The reineckate derivative of acetyldimethylethanolamine hydrochloride was also prepared and recrystallized from acetone-water. The decomposition point of this compound was observed to be about 182°. No literature values on the decomposition point of this compound could be found.

Mono Methyl Disodium Phosphate. The procedure for the preparation of mono methyl disodium phosphate of Bailly[99] was slightly modified in this work

A 0.5 N solution of Na_3PO_4 was enclosed in a pressure-resistant bottle. To one hundred milliliters of this solution was added 7.1 grams of methyl iodide and the flask closed securely by means of a rubber stopper held in place by a metal clamp. The reaction flask was agitated continuously at 65°C in an air bath, fitted out with a heating devise and a thermostat. After several hours the methyl iodide had completely disappeared. At this time the liquid was cooled and diluted to 125 milliliters. To the reaction mixture was then added the calculated amount of $BaCl_2.2\ H_2O$ necessary to precipitate the total uncombined phosphate. A sufficient amount of sodium hydroxide was then added to adjust the pH to the end point of phenolphthalein. The precipitated barium phosphate was filtered off. 4.36 grams of anhydrous $CaCl_2$ was added to the filtrate, and a precipitate of calcium mono methyl phosphate appeared immediately. An equivalent amount of sodium carbonate was added, and the solution boiled. The precipitated barium carbonate was filtered off and the solution concentrated until the sodium salt started precipitating. The mixture was set aside in a refrigerator. The sodium salt was precipitated completely by the addition of fifty milliliters of acetone. The crystals were filtered on a funnel and dried over anhydrous calcium chloride in vacuo.

By titrating a weighed amount of this compound with a standard solution of hydrochloric acid to the methyl orange

endpoint, the neutral equivalent was obtained. Results of this titration are given in Table 6.

TABLE 6. Titration of monomethyl disodium phosphate with 0.10 N hydrochloric acid to methyl orange end point.

weight of sample	milliliters 0.10 N HCl	neutral equivalent
0.1527 g	9.50 ml	160
0.1330	8.22	162
	AVERAGE	161

Neutral Equivalent: Calculated for $CH_3Na_2PO_4$, 156
Found, 161

<u>α-Keto-γ-methiolbutyric Acid</u>. The preparation of this keto acid followed the method devised by Meister[100] for the preparation of α-keto-β-methylvaleric acid.

22.8 grams of DL-methionine (U. S. Industrial Chemicals product) was dissolved in 750 milliliters of 0.05 M pyrophosphate buffer in a three-liter three-neck round bottom flask. The flask was immersed in a constant temperature bath at 37.0°C. A d-amino oxidase preparation was made from commercial acetone hog kidney powder by extracting the acetone powder with twenty volumes of ice cold water in a Waring blendor. The extract was then centrifuged for five minutes and filtered through gauze. 650 milliliters of this clear, yellow hog kidney extract was added to the methionine solution and the mixture stirred continuously for sixteen hours. A continuous stream of oxygen was bubbled through the mixture during the entire period of incubation. Capryl alcohol was added as needed to prevent foaming.

At the end of the incubation, the mixture was acidified to pH 4.5 with concentrated hydrochloric acid, heated with norit at 60-70°C, and filtered. The filtrate was evaporated in vacuo on a steam cone to about one-third its original volume. The solution was treated again with norit and filtered. The filtrate was then acidified with concentrated hydrochloric acid to pH 1.0. This filtrate was extracted eight times with equal volumes of ether. The ether extracts were dried for two hours over anhydrous magnesium sulfate, and the ether was removed by distillation. The keto acid was first distilled under vacuum using a carbon flame.

Two subsequent vacuum distillations were carried out. The following boiling point ranges and their respective pressures were observed:

distillation	temperature °C	pressure mm-Hg
second	85-92	0.8 - 1.1
third	75-78	0.5 - 0.6

Analysis: Calculated for $C_5H_8O_3S$: Carbon, 40.53%; hydrogen, 5.44%

Found: Carbon, 40.51, 41.14%; hydrogen, 6.71, 6.82% *

The 2,4 dinitrophenylhydrazone of the keto acid was prepared and recrystallized twice from methanol-water. The melting point was observed to be 138°C.

Analysis: Calculated for $C_{11}H_{12}O_6N_4S$: Nitrogen, 17.07%

Found: Nitrogen, 16.46, 16.51%

*The analyst reported some difficulty with the determination due to an explosion of the sample during the analysis just preceding this analysis.

Isolation of Choline From Rat Liver. A rat was killed by stunning and the liver removed immediately to be homogenized in a Waring glass blendor with sixty milliliters of 0.9% potassium chloride to which 17.2 milligrams of magnesium sulfate had been added. Fifty milliliters of the homogenate was made acid with concentrated hydrochloric acid, so that the final concentration of hydrochloric acid was 7.8%. The mixture was refluxed for twenty-four hours and was then treated with norite and filtered. To the clear filtrate was added an equal volume of saturated ammonium reineckate solution and the flask placed in an ice bath. An immediate pink precipitate formed which after half an hour was filtered and recrystallized twice from acetone and water. The reineckate was dried in vacuo at 100°C. 27.1 milligrams of choline reineckate was isolated from 4.65 grams of rat liver.

Analysis: Calculated for $C_9H_{20}ON_7CrS$: Nitrogen, 23.20%.

Found: Nitrogen, 22.37, 22.33%.

Another ten milliliters of the homogenate was treated with five milliliters of 10% trichloroacetic acid in order to precipitate all protein. The precipitated protein was filtered off, the filtrate was concentrated in vacuo and dried in vacuo over P_2O_5. The residue was dissolved in four milliliters of water, the mixture was filtered, and an equal volume of saturated ammonium reineckate added. No precipitate formed, proving the absence of any detectable amounts of free choline in about one gram of rat liver.

EXPERIMENTAL PROCEDURE IN THE USE

OF THE WARBURG RESPIROMETER

Instrument. The instrument consists of a rectangular constant temperature bath and the appropriate glass ware, manufactured by the American Instrument Company. The temperature range of the instrument is from room temperature to 50°C. The temperature is controlled by a bimetallic thermoregulator. The instrument is fitted with a shaking device for fourteen micromanometers. The rate of shaking can be controlled, and the optimum rate is ninety cycles per minute.

Reaction Flasks. The standard reaction flask is fitted with single side arm and center well; its volume is approximately 15 milliliters (Figure 3).

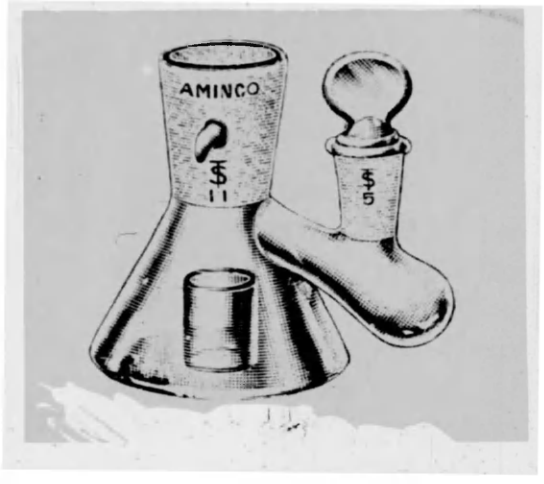


Figure 3. Warburg reaction flask

Large flasks with an approximate volume of 130 milliliters are only fitted with a center well and no side arm.

Care of Equipment. The flasks are cleaned thoroughly immediately after each run. They are first washed with benzene to dissolve the grease used on the joints. The flasks are then immersed in a hot hydrochloric nitric acid bath, rinsed five times with tap water, three times with distilled water, and twice with glass distilled water. It was found safer to clean flasks in the acid bath than to use dichromate cleaning solution, as any slight trace of chromium ions will poison many enzyme systems.

Calibration of Flasks. The flasks are calibrated by the method of Vogler and Krebs. The method is described in detail by Unbreit[101]. The calibrated flask volumes are given in Table 7.

Preparation of Animal Tissue. Two techniques for the preparation of animal tissue were used in this work, homogenate technique and slice technique.

A. Homogenate Technique. The animal is killed by stunning, and the organ to be homogenized is removed as fast as possible. In the majority of experiments in this work the liver was used. The organ is rinsed with cold distilled water, weighed, and put into the homogenizer tube containing Krebs-Ringer isotonic phosphate solution, ten times the weight of the tissue. The preparation of the Krebs-Ringer phosphate solution is described by Umbreit[102]. In a few experiments Krebs-Henseleit[103] solution was substituted for Krebs-Ringer solution. Krebs-Henseleit solution contains all of the components of Krebs-Ringer solution with the

TABLE 7. Calibrated volume of Warburg reaction flasks.

Flask Number	Volume in milliliters
1	14.6
2	12.2
3	11.8
4	11.8
5	14.6
6	11.9
7	14.0
8	15.2
9	12.0
10	12.5
11	12.6
12	12.0
13	13.1
14	12.4
1 (large)	140.8
2 (large)	140.0
3 (large)	135.1
4 (large)	130.0

exception of phosphate buffer and in addition contains glucose, sodium citrate and sodium bicarbonate.

In a few experiments, involving the use of cobalt ions, a phosphate-free Krebs-Ringer solution was employed.

As a homogenizer either a Waring blendor with a stainless-steel blade was used or an all-glass homogenizer. The all-glass homogenizer consists of a test tube (20 by 200 millimeter) and a close fitting power-driven pestle (Figure 4). The pestle is fitted out with six or seven sharp cutting teeth at the bottom; these cutting teeth are made by sealing small glass beads to the bottom of the pestle, then grinding them on an emery wheel so that each bead has a flat surface.

The homogenizer tube is kept in an ice bath while the tissue is being ground. With liver this operation usually did not take longer than one or two minutes. The homogenate was used immediately and measured with a Mohr pipet, having an enlarged opening.

B. Slice Technique. The top of a Petri dish is frosted with emory powder. A microscope slide is similarly treated. A three-hole variety razor blade is broken in half, lengthwise, and is held in place by a holder made from a piece of brass pipe, six millimeter in diameter and ten centimeter long. A two centimeter slit is sawed lengthwise on one end of the pipe, and a small threaded hole is made one centimeter from that end. A brass screw is fitted into that hole, and the one-half of the razor blade is clamped into the slit where it is fastened with the screw (Figure 5).

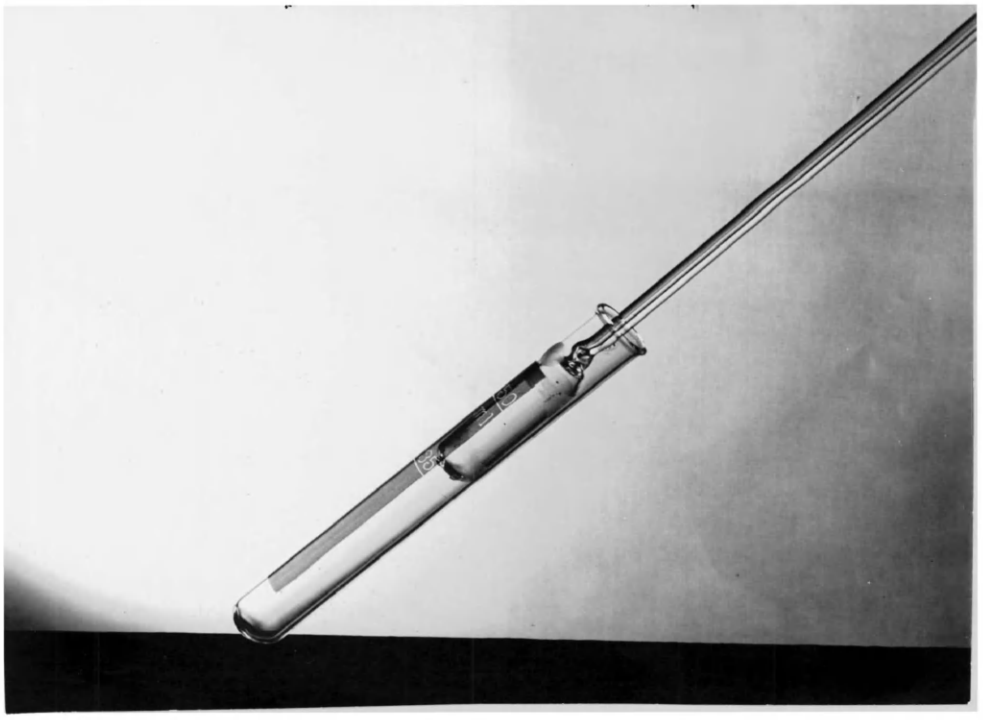


Figure 4. All-glass homogenizer

Figure 5. Instruments for tissue-slice technique

After the liver has been removed from a freshly killed animal, a small, approximately one square centimeter piece of the organ is placed on a two square centimeter piece of filter paper on top of the frosted side of the Petri dish. The blade is then moistened with Krebs-Ringer solution, the frosted side of the slide is placed on top of the tissue and a slight, varying amount of pressure is applied. The blade is drawn through the organ by guiding the blade along the surface of the slide. If the writing on the razor blade can be read with ease, the slice is of the correct thickness. The slices are transferred to a dish with Krebs-Ringer phosphate solution. The slices are then transferred as rapidly as time will permit to the reaction flasks.

Preparation of Substrates. Methionine was dissolved in McIlvaine's buffer of pH 7.2. The buffer consisted of 0.1 M citric acid and 0.2 M disodium phosphate. The solution was always freshly made for each run.

Ethanolamine was either used as the pure liquid, doubly distilled at 169-171°C, atmospheric pressure, or as a solution in McIlvaine's buffer of pH 7.2, unneutralized or neutralized to final pH 7.2 with dilute hydrochloric or phosphoric acids.

Adenosine triphosphate (ATP) was obtained as the sodium salt from Schwarz Laboratories, New York. It was either added directly to the homogenate or was used, dissolved in McIlvaine's buffer of pH 7.2.

Cytochrome-C was obtained from the VioBin Corporation as 0.5% solution in 0.9% sodium chloride water solution and used in this form.

Vitamin B_{12} was obtained from The Poultry Department, University of Maryland, in powder form and used in water solution. One gram of this powder contained one milligram of pure Vitamin B_{12}.

Diphosphopyridine nucleotide (DPN) was used in water solution.

Water used throughout this work was distilled in an all-glass apparatus.

Procedure of Actual Experiment. The substrates were measured into the Warburg flasks. In case "oxygen absorbed" was to be measured, 0.2 milliliter of 10% potassium hydroxide was pipetted into the center well whose upper surface was well greased with vaseline in order to prevent the potassium hydroxide from "creeping" over the side of the well. A two-square-centimeter-piece of filter paper (Whatman No. 1) was folded two or three times accordion-style and placed in the center well. The flasks were then connected to the manometers, making sure that the joints were well greased with vaseline. At this point the homogenate was added to the side arm, and the well greased tops placed on the side arms.

When slices were used, they were placed into the substrates in the flasks. After the flasks were then attached to the manometers, the assembly was placed as quickly as possible into the constant temperature bath. This technique suffered from the error that the reaction had already started before the first reading was made. A better procedure would have been to have placed the substrate in the side arm and the slices in the bottom of the flasks. In these experiments, however, the volume of the substrates was too large to be placed in the side arm whose capacity was about one milliliter.

With the large Warburg flasks which did not have a side arm, the method suffered from the same error, that the reaction could not be followed from its initial start.

After the manometers were assembled and placed into the water bath, they were shaken for ten minutes. The level of the fluid in the manometers was adjusted to the "zero" reading (usually 250 millimeters) and the stopcocks were closed. Placing one's thumb on top of the open arm of the manometer, the assembly was removed from the bath, inverted several times to about an angle of forty-five degrees, thereby mixing the homogenate and substrate, and returned to the bath as quickly as possible. Caution should be exercised at this time to prevent the flowing of the manometer fluid into the reaction flasks due to the change of temperature outside the bath.

After all homogenates were mixed, the shaker was started, and readings were taken at ten minute intervals, always returning the level of the fluid in the closed arm of the manometer to the zero reading. The readings were recorded on a convenient form.

After a set time, usually when the oxygen uptake in samples and blank had approached the same rate, the manometers were opened to the atmosphere, and the assemblies returned to their stand. The contents of each flask was poured into a volumetric flask of convenient size, the Warburg flasks were washed several times with distilled water, and the washings poured into the volumetric flasks. Five milliliters of a ten per cent trichloroacetic acid aqueous solution was then added to the contents of the volumetric flasks which were allowed to sit for thirty minutes. The precipitated

protein was filtered off, after the volumetric flasks had been filled to their mark. The filtrates were then ready for the various analyses.

The "Direct Method" for Carbon Dioxide[91]. Respiration can be measured as oxygen absorbed even if carbon dioxide is given off during the reaction, by absorbing all of the carbon dioxide in alkali placed in the center well. Therefore, if one has two flasks each respiring in exactly the same way, with the exception that carbon dioxide is absorbed in one flask and not absorbed in the other, one can calculate the amount of carbon dioxide liberated.

RESULTS AND CALCULATIONS

Sample Calculations of Run No. 11. Samples were incubated for five hours with fresh rat liver homogenate, prepared from one part liver and nine parts Krebs-Ringer phosphate solution. All substrates were dissolved in McIlvaine's buffer, pH 7.2, and ethanolamine solution was neutralized with dilute hydrochloric acid to final pH 7.2. Table 8 lists the contents of Flasks No. 1 and No. 5.

TABLE 8. Contents of Warburg flasks.

Flask Number	Contents	Volume of fluid (milliliters)
1	21.6 mg DL-methionine	
	1.0 mg ATP	
	20.0 mg ethanolamine	2.6
	1.0 ml homogenate	
	0.2 ml 10% KOH (center well)	
5	1.0 mg ATP	
	20.0 mg ethanolamine	
	1.0 ml homogenate	1.6
	0.2 ml 10% KOH (center well)	

The calculation of the flask constant is discussed in detail by Umbreit[93], whose method was used throughout this work. The flask constant represents a correction factor so that from observed pressure changes one can calculate the amount (in micro liters at 0°C and 760 mm pressure) of gas utilized or given off. The equation for the flask constant is:

$$(5.) \qquad k = \frac{V_g \frac{273}{T} + V_f a}{P_o}$$

V_g = volume of gas phase in flask including connecting tubes down to "zero" point

V_f = volume of fluid in flask

P_o = 760 mm Hg pressure, expressed in terms of manometer fluid which has a density of 1.0. P_o, therefore, is 760 x 13.60 (specific gravity of mercury) = 10,000

a = solubility of gas involved in liquid, expressed as milliliters of gas per milliliters of liquid, when gas is at a pressure of one atmosphere at temperature T. "a" for oxygen at 37° is equal to 0.024 ml O_2 per ml fluid at one atmosphere pressure.

Sample Calculations. By substituting values for Flask No. 1 in Equation (5.), the following expression is obtained:

$$k = \frac{(14,6000 - 2,600) \times \frac{273}{310} + 2,600 \times .0240}{10,000}$$

$$k = \frac{1057.6 + 62.4}{10,000} = 1.12$$

The data of Table 9 illustrate the method by which the manometer readings are recorded. The temperature of the bath was 37.0°.

TABLE 9. Record of manometer readings and flask constants.

Flask	1	5
k	1.12	1.18
Time (minutes)	Readings in millimeters	
0	250	250
10	241	248
20	226	239
30	213	234
40	196	227
50	182	220
60	170	214
70	152	204
120	93	170
120*	250	250
240	185	212
300	168	203

* Manometers were opened to the atmosphere and reset at manometer reading of 250.

The data of Table 10 illustrate the method of calculating the micro liters of oxygen uptake from the observed changes in the level of the manometer fluid of the blank and of the reaction flask manometer.

TABLE 10. Calculating oxygen uptake from manometer reading.

Total reading		Oxygen Uptake		Net oxygen uptake
Flask No. 1	Flask No. 5 (blank)	No. 1	No. 5	
in millimeters		in micro liters		in micro liters
239	127	268	150	118
(1)	(2)	(3)	(4)	(5)

Columns (1) and (2) are obtained by subtracting "final readings" from "initial readings", recorded in Table 9. Since the manometers were reset at 120 minutes, two such differences are obtained and are added subsequently to obtain "total reading" in millimeters. Columns (3) and (4) are calculated by multiplying the "total reading" of each flask by its respective flask constant, listed in Table 9. Column (5) is obtained by subtracting Column (4) from (3).

In order to calculate the number of moles of oxygen taken up, it is necessary to convert micro liters to milliliters and divide by 22,400. "Net oxygen uptake" is $\frac{118 \times 10^{-3}}{22,400}$ mols, which calculates to 5.27×10^{-6} moles O_2.

In order to determine the amount of methionine which had disappeared in the course of the reaction, it was necessary to obtain initial and final concentration of methionine. Aliquots of the sample before and after incubation with rat liver were analyzed for methionine by the previously described method of McCarthy and Sullivan[12]. The data of Table 11 illustrates the method for calculating the amount of methionine disappearing during the reaction.

TABLE 11. Per cent light transmission at 510 mu before and after incubation with rat liver. Concentration of methionine before and after incubation.

Sample No. 1	% Transmission	milligrams methionine	
		1/3 aliquot	total
before incubation	55.9	7.2	21.6
after incubation	57.9	6.6	19.8
	(1)	(2)	(3)

Column (2) for "before incubation" can be obtained directly from Figure 1, as only methionine is present, and Figure 1 is a plot of concentration of methionine against per cent transmission. This value can also be obtained by substituting "per cent transmission" in Equation (1.) as follows:

(1.) $- \log T = kcl$

$T = .559$

$k = .0350\ cm^{-1}mg^{-1}/100\ ml$

$l = 1.0\ cm$

$- \log .559 = .0350 \times c$

To solve for c :

$$c = \frac{.25259}{.0350} = 7.22\ mg/100\ ml\ solution$$

The same result is obtained directly from Figure 1.

In order to evaluate Column (2) for "after incubation", the simple Beer Lambert's equation cannot be used, as the solution now consists of a mixture of methionine and its keto analogue, α-keto-δ-methiolbutyric acid. Therefore, the value for the observed transmission and the initial methionine concentration must be substituted in Equation (3.) with the following results:

$$(3.) \quad - \log \frac{I}{I_o} = - \log T = k_1(c_o - c_2) + k_2c_2$$

Rearranging Equation (3.), the following equation is obtained:

$$(6.) \quad - \log T = k_1c_o + (k_2 - k_1)c_2$$

The following values are now substituted in Equation (6.):

$T = .579$

$k_2 = .0350;\ k_1 = .00811$

$c_o = 7.2$

$- \log .579 = .00811 \times 7.2 + (.0350 - .00811)c_2$

To solve for c_2, the following equation is obtained:

$$(7.) \quad c_2 = \text{concentration of methionine} = \frac{-\log .579 - (.00811 \times 7.2)}{.0269}$$

$$= 6.6\ mg/100\ ml\ solution$$

Values of Column (3) are calculated by multiplying the values in Column (2) by 3.

By subtracting the values in Column (3) the total amount of methionine which has disappeared can be calculated. The moles of methionine which ave disappeared are calculated by dividing "grams methionine" by 149.21, the molecular weight of methionine.

Table 12 is a summation of results of four different samples in which methionine was incubated with rat liver homogenate. From these results the ratio "methionine disappearing : oxygen uptake" can be calculated. The average value of this ratio for these samples calculated to be 2, which confirmed the previously discussed mode of action of the enzyme d-amino acid dehydrogenase.

TABLE 12. Calculating the ratio "methionine disappearing : oxygen uptake".

No.	oxygen uptake microliters			molx10^6	methionine disappeared		$\frac{\text{methionine}}{\text{oxygen}}$
	total	blank	net	net	mg	molx10^6	
1	268	150	118	5.27	1.8	12.1	2.3
3	244	166	78	3.48	0.9	6.1	1.8
4	268	166	102	4.55	1.8	12.1	2.7
6	213	128	85	3.79	0.6	4.0	1.1
						AVERAGE	2.0

Sample Calculations of Run No. 19. This run was designed so as to measure the amount of oxygen uptake and carbon dioxide production when methionine was incubated with rat liver. Flask No. 1 contained potassium hydroxide in the center well and Flask No. 7 did not. The following equation was developed by Umbreit[91] for the calculation of the amount of carbon dioxide production:

$$(8.) \quad X_{co_2} = (h - \frac{h'k'_{o_2}}{k_{o_2}})k_{co_2}$$

h' = manometer change in Flask No. 1 (with KOH)

h = manometer change in Flask No. 7 (without KOH)

X_{co_2} = micro liters of carbon dioxide produced

k'_{o_2} = flask constant of Flask No. 1 in respect to oxygen

k_{co_2} = flask constant of Flask No. 7 in respect to CO_2

k_{o_2} = flask constant of Flask No. 7 in respect to O_2

In order to calculate k_{co_2}, Equation (5.) is used, and the value for "a", the solubility of CO_2 at 37°C and one atmosphere pressure, is 0.57, The following table lists the necessary data in order to calculate the flask constants:

Flask No.	V_g	V_f
	micro liters	
1	14,600-2,200	2,200
7	14,000-2,000	2,000

By substituting the above values in Equation (5.), the following are obtained:

$$k_{co_2} = \frac{(14,000-2,000)\times\frac{273}{310} + 2,000 \times 0.57}{10,000}$$

$$= 1.22$$

$$k'_{o_2} = \frac{(14,600-2,200)\times\frac{273}{310} + 2,200 \times .0240}{10,000}$$

$$= 1.15$$

$$k_{o_2} = \frac{(14,000-2,000)\times\frac{273}{310} + 2,000 \times .0240}{10,000}$$

$$= 1.11$$

In this run h' was observed to be 294 mm and h 101 mm. After substituting all values necessary in Equation (8.), the following is obtained:

$$X_{co_2} = (101 - \frac{294 \times 1.15}{1.11}) \times 1.22 = - 248 \text{ micro liters}$$

The negative sign signifies carbon dioxide production. The amount of oxygen uptake can be calculated by multiplying h' by k'_{o_2}, which calculates to be 294 x 1.15 = 338 micro liters. Therefore the ratio of oxygen uptake over carbon dioxide production calculates to be:

$$\frac{338}{248} = 1.36$$

Run No. 1.

Rabbit muscle homogenate in Krebs-Ringer phosphate solution.

Freshly distilled ethanolamine.

DL-methionine in McIlvaine's buffer (pH 7.2).

Time of incubation: 17 hours.

No.	Contents	mols O_2 absorbed	mols methionine disappeared
4	54.0 mg DL-methionine		
	1.0 ml 10% homogenate		
	50.0 mg ethanolamine	0	0
	0.2 ml 10% KOH in well		
7	54.0 mg DL-methionine		
	1.0 ml 10% homogenate		
	50.0 mg ethanolamine	0	0
9	54.0 mg DL-methionine		
	1.0 ml 10% homogenate		
	0.2 ml 10% KOH in well	0	0

Run No. 2.

Rabbit liver homogenate in Krebs-Ringer phosphate.

Ethanolamine in McIlvaine's buffer, neutralized with dilute.

H_3PO_4 to final pH of 7.2.

Methionine and adenosine triphosphate dissolved in McIlvaine's buffer (pH 7.2).

Time of incubation: 7 hours, 15 minutes.

No.	Contents	mols O_2 absorbed X 10^6	mols methionine disappeared X 10^6
5	16.9 mg DL-methionine 1.0 ml 10% homogenate 1.0 mg ATP	3.36	0
3	32.7 mg L-methionine * 1.0 ml 10% homogenate 15.0 mg ethanolamine	2.57	2.02
4	35.6 mg D-methionine * 1.0 ml 10% homogenate 15.0 mg ethanolamine	2.57	0

Note: No blank was run for endogenous respiration.

* D-methionine and L-methionine from Dr. J. P. Greenstein, NIH, Cancer Institute, Bethesda, Maryland.

Run No. 3.

20% fresh rabbit liver homogenate in Krebs-Ringer phosphate.

DL-methionine in phosphate buffer (pH 7.38).

Ethanolamine in phosphate buffer, neutralized with dil. H_3PO_4 to final pH of 7.38.

Time of incubation: 4 hours.

No.	Contents	mol methionine disappearing	mol O_2 absorbed X 10^6
1	29.0 mg DL-methionine 40.0 mg ethanolamine 1.0 ml homogenate	0	0.6
5	29.0 mg DL-methionine 40.0 mg ethanolamine 1.0 ml homogenate 1.0 mg ATP	0	0
7	29.0 mg DL-methionine 1.0 ml homogenate 1.0 mg ATP	0	0
13	29.0 mg DL-methionine 40.0 mg ethanolamine 1.0 mg ATP 1.0 ml homogenate	0	0

Run. No. 4.

Fresh guinea pig liver homogenate in Krebs-Ringer phosphate.

All substrates dissolved in McIlvaine's buffer (pH 7.2).

Ethanolamine neutralized with dilute H_3PO_4 to final pH of 7.2 .

Time of incubation: 4 hours.

Sample	Contents	mols methionine disappearing	mol O_2 absorbed X 10^6
2	24.8 mg DL-methionine		
	40.0 mg ethanolamine		
	1.0 mg ATP		
	2 X 10^{-3} mg Vitamin B_{12}		
	1.0 ml 10% homogenate	0	0.9
7	24.8 mg DL-methionine		
	1.0 mg ATP		
	2 X 10^{-3} mg Vitamin B_{12}		
	1.0 ml 10% homogenate	0	2.9

Run No. 5.

Fresh rat liver slices.

DL-methionine dissolved in McIlvaine's buffer, pH 8.

Ethanolamine neutralized with dilute HCl to pH 8.0.

Ethanolamine, not neutralized

Time of incubation: 4 hours.

No.	Contents	Methionine disappeared milligrams	keto group A	keto group B	choline
1	38.0 mg DL-methionine 40.0 mg ethanolamine HCl	14.0	pos.	pos.	neg.
2	38.0 mg DL-methionine 50.0 mg ethanolamine	0.8	neg.	neg.	neg.

Tissue slices were homogenized immediately before methionine analysis in order to prevent a disappearance of methionine due to diffusion into the cell walls.

Choline was qualitatively analyzed by the addition of an equal volume of saturated aqueous ammonium reineckate solution.

Qualitative tests A and B for the keto group have been previously described.

Run No. 6.

Fresh rat liver slices.

DL-methionine dissolved in McIlvaine's buffer, pH 7.2.

Reaction flasks were saturated with oxygen during entire run.

Time of incubation: 3 hours.

Sample No.	Contents	2,4 dinitro-phenylhydrazone	choline analysis
1	74.5 mg DL-methionine 200 mg ethanolamine, neutralized with HCl to pH 7.2	positive	negative
2	74.5 mg DL-methionine	positive	negative
3	74.5 mg DL-methionine 200 mg ethanolamine	negative	negative

2,4 dinitrophenylhydrazone was recrystallized from alcohol water. The melting point was observed to be 137°.

Analysis: Calculated for $C_{11}H_{12}N_4O_6S$: Nitrogen, 17.07%

Found: Nitrogen, 16.45%

Run. No. 7.

298 milligrams of DL-methionine dissolved in 20.0 milliliters of McIlvaine's buffer, pH 8, was incubated at 37°C for nineteen hours with fresh rat liver slices. At the end of the incubation period, the sample was filtered and 50.0 milliliters of water added to the clear filtrate. 15.0 milliliters of 2,4 dinitrophenylhydrazine reagent, made up as a saturated solution in 2 N hydrochloric acid, was added, and an immediate yellow precipitate appeared. The flask was set aside in a refrigerator for one hour and the precipitate filtered on a funnel. The precipitate was recrystallized twice from an alcohol water solution, and then dried overnight in vacuo at 100°C. The melting point was observed to be 147-148°C. The reported literature value for the melting point of the 2,4 dinitrophenylhydrazone of methionine is 149°C.[83,85]

Analysis: Calculated for $C_{11}H_{12}N_4O_6S$: Nitrogen, 17.07%
Found: Nitrogen, 16.35%

The discrepancy between the calculated and found percentage of nitrogen does not appear too serious when comparing this result with others in the literature. Waelsch[83] found 16.57% nitrogen, compared to 16.35% in this work. The small amount of the derivative isolated in this experiment did not permit any further recrystallizations or a second nitrogen analysis.

Run No. 8.

Fresh rat liver slices.

DL-methionine dissolved in McIlvaine's buffer (pH of 8.0).

Ethanolamine neutralized with dilute HCl.

Stream of dry nitrogen flushed through samples continuously.

Time of incubation: 3 hours.

Sample No.	Contents	methionine start	methionine end
1	DL-M ethanolamine	34.4 mg	35.5 mg
2	DL-M	34.4	34.8

Both samples gave negative results with qualitative test A for testing the presence of keto groups.

Run No. 9.

This experiment was designed to test the activity of hog kidney powder.

153.8 mg of DL-methionine, dissolved in 10.0 ml of McIlvaine's buffer (pH 8.0) was incubated for twenty hours with 250 milligrams of acetone hog kidney powder (Vio-Bin Corporation product). The flask was shaken continuously in a constant temperature bath at 38°C. At the end of incubation, the sample was deproteinized by the addition of 5.0 milliliters of 10% trichloroacetic acid. The sample was filtered and analyzed for methionine.

Dl-methionine at start mg	DL-methionine at end mg
153.8	127.5

The above experiment was repeated, using different amounts of methionine. Samples were incubated with shaking at 38°C for 6½ hours.

Sample No.	DL-methionine at start (mg)	DL-methionine at end (mg)
1	72.5	56.0
2	36.25	28.0
3	36.25	28.5

Run No. 10. (Non-Enzymatic Run)

DL-methionine and ethanolamine dissolved in water.

Flask was incubated at 37°.

Period of incubation: six days.

No.	Contents	O_2 absorbed		methionine disappeared			free choline
		ml x 10^3	mol x 10^6	% T mu	mg	mol x 10^6	
	1.00 g DL-methionine				0	0	0
	1.00 g ethanolamine						

Run No. 11.

Fresh rat liver homogenate in Krebs-Ringer phosphate.

DL-methionine dissolved in McIlvaine's buffer (pH 7.2).

Ethanolamine neutralized with dilute HCl to pH 7.2.

Adenosine triphosphate (ATP) dissolved in McIlvaine's buffer.

Time of incubation: 5 hours.

No.	Contents	mols methionine disappearing X 10^6	mols O_2 absorbed X 10^6	methionine: oxygen
1	21.6 mg DL-methionine 20.0 mg ethanolamine 1.0 mg ATP 1.0 ml 10% homogenate	12.10	5.27	2.3
3	21.6 mg DL-methionine 1.0 mg ATP 1.0 ml 10% homogenate	6.10	3.48	1.8
4	21.6 mg DL-methionine 1.0 mg ATP 1.0 ml 10% homogenate	12.10	4.55	2.7
6	21.6 mg DL-methionine 20.0 mg ethanolamine 1.0 mg ATP 1.0 ml 10% homogenate	4.00	3.79	1.1

Run No. 12.

Fresh rat liver homogenate in Krebs-Ringer phosphate.

DL-methionine dissolved in McIlvaine's buffer (pH 7.2).

Adenosine triphosphate (ATP) in McIlvaine's buffer.

Ethanolamine neutralized with dilute phosphoric acid to pH 7.2.

Time of incubation: 10 hours.

Sample No.	Contents	mols methionine disappearing X 10^6	mols O_2 absorbed X 10^6	methionine: oxygen
8	35.8 mg methionine 1.0 ml 5% homogenate	16.5	5.2	3.2
10	35.8 mg methionine 1.0 ml 5% homogenate 40.0 mg ethanolamine 2.0 mg ATP 0.25 ml cytochrome-C	25.3	11.7	2.2
11	35.8 mg methionine 1.0 ml 5% homogenate 40.0 mg ethanolamine 2.0 mg ATP 0.25 ml cytochrome-C	16.5	9.3	1.8

Run No. 13.

Fresh rat liver homogenate in Krebs-Ringer phosphate .

All substrates dissolved in McIlvaine's buffer (pH 7.2).

Time of incubation: 7 hours.

Sample No. (large flasks)	Contents	mols methionine disappearing X 10^6	mols O_2 absorbed X 10^6	methionine oxygen
1	60.8 mg DL-methionine 40.0 mg ethanolamine 1.0 mg ATP 5.0 ml 10% homogenate	83.0	43.0	1.93
2	60.8 mg DL-methionine 1.0 mg ATP 5.0 ml 10% homogenate	83.0	42.5	1.95
3	60.8 mg DL-methionine 30.8 mg glycocyamine 1.0 mg ATP 5.0 ml 10% homogenate	42.4	33.5	1.27

Sample No.	Sulfhydryl Analysis (Method "A")
1	negative
2	negative
3	negative

Run No. 14.

Fresh rat liver homogenate in Krebs-Ringer phosphate.

DL-methionine in McIlvaine's buffer, pH (7.2).

ATP in McIlvaine's buffer, pH (7.2).

DL-serine in McIlvaine's buffer.

Ethanolamine neutralized with dilute HCl (pH 7.2).

Time of incubation: 3 hours.

Sample No.	Contents mol x 10^3	mg methionine disappeared	percentage methionine disappeared
1	0.25 DL-methionine 0.04 ethanolamine 0.25 ATP	4.2	8.7
2	0.25 DL-methionine 0.13 DL-serine 0.25 ATP	2.1	4.7
3	0.25 DL-methionine 0.25 ATP	1.4	2.7

Run No. 15.

Fresh rat liver homogenate in Krebs-Henseleit solution.

Choline chloride dissolved in Krebs-Henseleit solution.

$CoCl_2$ dissolved in water.

All samples contained 1.0 milligram of ATP.

Time of incubation: 90 minutes.

Contents	Sample No.	oxygen absorbed milliliters X 10^3
10.0 mg choline chloride 1.0 ml 10% homogenate	1	50.0
	2	38.5
	3	25.7
	AVERAGE	38.1
10.0 mg choline chloride 1.0 ml 10% homogenate 10^{-3}M $CoCl_2$	4	27.0
	5	27.4
	6	26.4
	AVERAGE	26.9

The samples which contained Co^{++} ions showed an average of 29.4% inhibition in oxygen uptake over those samples which did not contain Co^{++}.

Run No. 16.

Fresh rat liver homogenate in Krebs-Ringer phosphate-free solution.

Choline chloride dissolved in Krebs-Ringer solution.

$CoCl_2$ dissolved in Krebs-Ringer solution.

Time of incubation: 90 minutes.

Contents	Sample No.	oxygen absorbed milliliters X 10^3
4.0 mg choline chloride 1.0 ml 10% homogenate 1.0 mg ATP	1	
	1	22.0
	2	3.6
	3	5.5
4.0 mg choline chloride 1.0 ml 10% homogenate 1.0 mg ATP 10^{-3}M $CoCl_2$	4	5.5
	5	5.5
	6	3.7

If the results of Sample No. 1 are disregarded, no inhibition in oxygen uptake could be observed in the samples containing Co^{++} ions.

Run No. 17.

Five samples containing 24.5 milligrams of DL-methionine and 40.0 milligrams of ethanolamine were incubated with fresh rat slices for nineteen hours. DL-methionine was dissolved in McIlvaine's buffer, pH 7.2, and the ethanolamine was neutralized with dilute phosphoric acid to final pH 7.2. At the end of the incubation period, aliquots of all five samples were analyzed for free sulfhydryl groups by Method B. No free sulfhydryl groups were detected in any sample.

Run No. 17-A.

Fresh rat liver homogenate in Krebs-Ringer phosphate-free solution

Aliquots from Run No. 17 used as substrates

Time of incubation: 6 hours

Contents	No.	oxygen absorbed milliliters X 10^3	
		10^{-3}M Co^{++}	without Co^{++}
One-third aliquots from Run No. 17 1.0 mg ATP 1.o ml 10% homogenate	1	53	
	3	43	
	5	86	
	2		200
	4		251
	6		286
	AVERAGE	61	246

Samples containing Co^{++} showed 75% inhibition of oxygen uptake over those samples which did not contain Co^{++}.

Run No. 18.

Fresh rat liver homogenate in Krebs-Ringer phosphate-free solution.

DL-methionine dissolved in McIlvaine's buffer (pH 7.2).

Ethanolamine neutralized with dilute HCl to pH 7.2.

All samples contained 1.0 milligram of ATP.

Time of incubation: one hour.

Contents	No.	oxygen absorbed milliliters X 10^3	
		10^{-3}M Co^{++}	without Co^{++}
13.0 mg DL-methionine 40.0 mg ethanolamine 1.0 ml 10% homogenate	5	12.5	
	6		20.4
13.0 mg DL-methionine 1.0 ml 10% homogenate	7	0	
	8		30.7
10.0 mg choline chloride 1.0 ml 10% homogenate	13	153.5	
	14		113.1

Run No. 18-A.

Fresh rat liver homogenate in Krebs-Ringer phosphate solution.

All substrates dissolved in McIlvaine's buffer (pH 7.2).

All samples contained 1.0 milligram of ATP.

Time of incubation: 16 hours.

Contents	No.	oxygen absorbed mols X 10^6		methionine disappeared mols X 10^6
		10^{-3}M Co^{++}	no Co^{++}	
65.0 mg DL-methionine 92.0 mg acetyldimethyl-ethanolamine 5.0 ml 10% homogenate	1(large)	10.0		0
	2(large)		31.6	30.2
65.0 mg DL-methionine 5.0 ml 10% homogenate	3(large)	32.2		40.2
	4(large)		36.6	50.3

To aliquots of Samples Nos. 1 and 2 were added equal volumes of ammonium reineckate solution, and no immediate precipitate appeared. It was, therefore, concluded that no detectable amounts of choline had been formed.

Run No. 19.

Fresh rat liver slices.

DL-methionine dissolved in McIlvaine's buffer (pH 7.2).

All flasks contained 21.70 mg DL-methionine and 100 mg slices.

Time of incubation: 5 hours, 20 minutes.

with KOH		without KOH		
No.	oxygen absorbed milliliters X 10^3	No.	carbon dioxide produced milliliters X 10^3	$\frac{\text{oxygen}}{CO_2}$
1	338	7	248	1.36
4	226	9	112	2.02
5	181	10	110	1.64
6	187	11	79	2.36
			AVERAGE	1.85

Run No. 20.

Fresh rat liver slices.

DL-methionine dissolved in McIlvaine's buffer (pH 7.2).

Ethanolamine neutralized with dilute H_3PO_4 to pH of 7.2.

Time of incubation: 6 hours.

All flasks contain 22.45 mg methionine, 40.0 mg ethanolamine, and 100 mg liver slices.

with KOH		without KOH	
No.	ul O_2	No.	ul O_2
1	115.4	6	187.6
2	123.4	7	137.1
3	101.4	8	132.6
4	90.4	9	110.6
5	148.4	10	113.6
AVERAGE	135.4		136.4

Run No. 21.

Fresh rat liver homogenate in Krebs-Henseleit solution.

Monomethyl disodium phosphate neutralized with dilute HCl to pH 7.0.

Ethanolamine neutralized with dilute HCl to pH 7.0.

$CoCl_2$ dissolved in water.

Time of incubation: 70 minutes.

Contents	Sample No.	oxygen absorbed milliliters X 10^3
14.0 mg $CH_3Na_2PO_4$ 40.0 mg ethanolamine 1.0 ml 10% homogenate	2	8.0
	3	0
	4	-2.0
14.0 mg $CH_3Na_2PO_4$ 40.0 mg ethanolamine 1.0 ml 10% homogenate 10^{-3}M $CoCl_2$	5	4.0
	6	2.0
	7	11.0

Run No. 22.

Fresh rat liver homogenate in 0.06 M $NaHCO_3$.

Reaction flasks constantly kept under atmosphere of 5% CO_2-95% N_2.

Disodium methylphosphate and ethanolamine neutralized with CO_2 to pH 7.

5 mg ATP added to each sample.

Time of incubation: 5 hours.

Flask No.	Contents	Free Choline
1	150 mg $Na_2CH_3PO_4$ 200 mg ethanolamine 5.0 ml 10% homogenate	negative
2	do. do.	negative

At the end of the incubation period, both samples were deproteinized with trichloroacetic acid and to the clear filtrate equal volumes of ammonium reineckate solution added. No precipitate formed.

Run No. 23.

Fresh rat liver homogenate in Krebs-Henseleit solution.

DL-methionine dissolved in Krebs-Henseleit solution.

Ethanolamine neutralized with dilute HCl to pH 7.2.

$CoCl_2$ dissolved in water.

Time of incubation: One hour.

Contents	No.	oxygen absorbed milliliters X 10^3
12.8 mg DL-methionine		
40.0 mg ethanolamine	2	41.3
1.0 ml 10% homogenate	3	36.3
	4	33.8
AVERAGE		37.1
12.8 mg DL-methionine		
40.0 mg ethanolamine	5	42.8
10^{-3}M $CoCl_2$	6	37.8
	7	40.8
AVERAGE		40.4

Five milliliters of 10% trichloroacetic acid was added to each sample at the end of the incubation period. The samples were filtered after half an hour and the combined filtrates were treated according to directions by Barrenscheen[62] for the isolation of choline.

The filtrate was washed four times with an equal volume of ether, and an equal volume of 95% ethanol added to the washed filtrate. The

solution became cloudy due to the precipitation of glycogen and was filtered. The filtrate was concentrated in vacuo at 40°, the residue was dissolved in 3.0 milliliters of water, and an equal volume of saturated ammonium reineckate solution added. A silvery precipitate appeared immediately. The flask was heated on a water bath at 60°C for thirty minutes, the precipitate collected by filtration and washed once with 1.0 milliliter of saturated ammonium reineckate solution. Attempts to recrystallize the precipitate from acetone-water were unsuccessful. The precipitate was dried in vacuo at 100°, and the decomposition point found to be about 170°.

Analysis: Calculated for $C_6H_{14}N_7OS_4Cr$: C, 18.95; H, 3.68; N, 25.79.

Found: C, 18.91; H, 3.72; N, 25.04.

It was, therefore, concluded that the precipitate was ethanolamine reineckate.

Run No. 24.

Fresh rat liver homogenate in Krebs-Ringer phosphate solution.

All substrates dissolved in McIlvaine's buffer (pH 7.2).

Eserine (physostigmine) dissolved in water.

Acetyldimethylethanolamine hydrochloride neutralized with 10% KOH to pH 7.2.

Time of incubation: 280 minutes.

Sample No.	Contents
1	53.1 mg DL-methionine
2	68.0 mg acetyldimethylethanolamine.HCl
	10^{-6}M eserine
	1.0 mg ATP
	5.0 ml 10% homogenate
3	53.1 mg DL-methionine
	68.0 mg acetyldimethylethanolamine.HCl
	1.0 mg ATP
	5.0 ml 10% homogenate

After the incubation period all samples were deproteinized with 10% trichloroacetic acid, and after standing for half an hour the samples were filtered. To each filtrate was added an equal volume of saturated ammonium reineckate solution and a pink precipitate formed at once in each sample. The decomposition point of the precipitate was found to be 182°C (with decomposition).

The data in Table 13 compare the melting points or decomposition points of several reineckates.

TABLE 13. Melting points or decomposition points of several reineckates.

Substance	Melting point
unknown reineckate	182°C (with decomposition)
acetyldimethylaminoethanol reineckate	183-185°C (with decomposition)
acetylcholine reineckate	146°C

From these observations it would seem that the unknown reineckate was acetyldimethylmethylaminoethanol reineckate and not acetylcholine reineckate.

Run No. 25.

Fresh rat liver homogenate in Krebs-Ringer phosphate solution.

DL-methionine and DL-serine in Krebs-Ringer phosphate solution.

1.0 mg ATP and 0.1 ml of 0.5% cytochrome-C added to all samples.

Time of incubation: 90 minutes.

No.	Contents	oxygen absorbed milliliters X 10^3	methionine disappeared mols X 10^6
1	14.1 mg methionine 10.0 mg serine	29.7	0
2	14.1 mg methionine 10.0 mg serine	(leak)	0
3	14.1 mg methionine	0	2.68
4	14.1 mg methionine	0	5.36

All samples contained 1 milliliter of 10% homogenate

Run No. 27.

Fresh rat liver homogenate in Krebs-Henseleit solution.

All substrates dissolved in Krebs-Henseleit solution.

Ethanolamine neutralized with dilute HCl to pH 7.2.

All samples contained 1.0 milliliter of 10% homogenate.

Time of incubation: 3 hours.

No.	Contents	oxygen absorbed mols X 10^6	methionine disappeared mols X 10^6
3	11.6 mg methionine 7.0 mg serine	6.25	6.7
6	11.6 mg methionine 40.0 mg ethanolamine	2.28	0
8	11.6 mg methionine	4.70	0.7
9	11.6 mg methionine	2.68	0

Run No. 28.

Fresh rat liver homogenate in Krebs-Henseleit solution.

All substrates dissolved in Krebs-Henseleit solution.

Each sample contained 5.0 mg ATP

Ethanolamine neutralized with dilute HCl to pH 7.2.

Time of incubation: 19 hours.

No.	Contents	milligrams methionine disappeared	milligrams choline
1, 2	59.3 mg methionine 30.0 mg serine 5.0 ml 10% homogenate	0	0.90
3, 4	59.3 mg methionine 200 mg ethanolamine 5.0 ml 10% homogenate	2.3	0.60
6	59.3 mg methionine 5.0 ml 10% homogenate	0.9	
Blank	5.0 ml 10% homogenate		1.15

The samples which were analyzed for choline quantitatively were hydrolyzed by hydrochloric acid with a final concentration of 7.8%. These samples were refluxed twenty-four hours.

Run No. 29.

Fresh rat liver homogenate in Krebs-Ringer phosphate.

L-methionine and DL-serine in McIlvaine's buffer (pH 7.2).

Cephalin dissolved in dioxane.

Ethanolamine neutralized with dilute HCl to final pH of 7.2.

All samples contained 5.0 mg adenosine triphosphate.

Time of incubation: 17 hours.

No.	Contents	mg methionine disappearing	Test B for α-keto acids
1	30.75 mg L-methionine 40.0 mg ethanolamine 5.0 ml 10% homogenate	0	negative
2	30.75 mg L-methionine 14.2 mg DL-serine 5.0 ml 10% homogenate	0	negative
3	30.75 mg L-methionine 50.0 mg cephalin 5.0 ml 10% homogenate	1.0	positive
4	30.75 mg L-methionine 5.0 ml 10% homogenate	0	negative
6	30.75 mg L-methionine 50.0 mg unneutralized ethanolamine 300 mg liver slices	0.75	negative

Upon the addition of an equal volume of saturated ammonium reineckate to the protein-free filtrate of Sample No. 3, a pink precipitate appeared. This precipitate was found to be very water soluble. When it was found that ethanolamine reineckate was also soluble in water but that choline and lecithin reineckates were not, it was concluded that the reineckate from Sample No. 3 must have been ethanolamine reineckate, the ethanolamine being formed by the hydrolysis of cephalin.

SUMMARY

1. The biological synthesis of choline has been studied. Previous claims for the formation of choline from methionine and ethanolamine by in-vitro experiments were based on the disappearance of methionine, analyzed by the McCarthy-Sullivan method[12], or on the formation of choline, precipitated as choline reineckate. Present experiments have proved that the "disappearance of methionine" actually represented the formation of α-keto-γ-methiolbutyric acid, the keto analogue of methionine. It was also established that the reported "choline reineckate" actually was ethanolamine reineckate. Serious doubt is, therefore, cast on the reported evidence of choline formation from methionine and ethanolamine by in-vitro reactions.

2. Evidence for choline formation by rat liver tissue was obtained by the use of Co^{++} ions which inhibit the activity of the enzyme choline oxidase.

3. Evidence was obtained which indicated that the "labile" methyl group of methionine was oxidized enzymatically to CO_2 in the absence of ethanolamine by rat liver tissue.

4. A series of methyl donors and methyl acceptors other than methionine and ethanolamine exhibited no choline-forming properties.

5. A new method to demonstrate the stoichiometric relationship between methionine and oxygen in the enzymatic deamination of methionine has been developed. It has been shown that two

molecules of methionine react with one molecule of oxygen.

APPENDIX

ADDITIONAL EXPERIMENTAL DATA

	No.	Manometer reading (millimeters)		flask constant		Methionine determination	
		sample	blank	sample	blank	aliquot	% T
Run No. 2							
	3	84	-	.69	-	1/3	44.1
	4	84	-	.69	-	2/15	68.2
	5	81	-	.93	-	2/15	62.2,62.5
Run No. 3							
	1	92	91	.97	.83	2/15	70.5
	5	115	188	.87	1.11	2/15	70.4
	7	170	188	.91	1.11	2/15	71.2
	13	113	188	.71	1.11	2/15	71.7
Run No. 4							
	2	231	179	.76	.88	2/15	71.5
	7	217	179	1.00	.88	2/15	73.5,73.9
Run No. 5							
	1	not measured				1/10	81.3
	2	" "				1/10	79.2,78.5
Run No. 8							
	1	not measured				1/10	75.1
	2	" "				1/10	74.5
Run No. 9							
	1	not measured				1/20	77.5
	2	" "				1/10	77.5
	3	" "				1/10	77.1
Run No. 11							
	1	239	127	1.12	1.18	1/3	57.9
	3	274	159	0.89	1.04	1/3	57.5,57.3
	4	302	159	0.89	1.04	1/3	58.2
	6	248	129	0.86	0.99	1/3	56.5,56.9
Run No. 12							
	8	102	-	1.14	-	2/15	69.5
	10	358	-	.69	-	2/15	70.5;70.9 70.3;69.5
	11	207	-	.93	-	2/15	69.5

	No.	Manometer reading (millimeters)		flask constant		Methionine determination	
		sample	blank	sample	blank	aliquot	% T
Run No. 13							
	1	86	-	11.2	-	2/25	71.5
	2	84	-	11.3	-	2/25	71.5
	3	72	-	10.4	-	2/25	70.8
Run No. 14							
	1	not measured				1/5	58.2
	2	"	"			1/5	56.7
	3	"	"			1/5	56.2
Run No. 15							
	1	88	40	1.00	.95		
	2	96	40	.79	.95		
	3	84	40	.76	.95		
	4	76	29	.76	1.06		
	5	58	29	1.00	1.06		
	6	73	29	.77	1.06		
Run No. 16							
	1	104	94	1.01	.95		
	2	108	94	.79	.95		
	3	112	94	.76	.95		
	4	67	55	.76	1.06		
	5	61	55	1.01	1.06		
	6	77	55	.77	1.06		
Run No. 17-A							
	1	184	157	.99	.83		
	2	181	-75	.78	.78		
	3	228	157	.76	.83		
	4	256	-75	.76	.78		
	5	218	157	1.00	.83		
	6	298	-75	.77	.78		
Run No. 18							
	5	30	20	.97	.83		
	6	51	20	.75	.89		
	7	16	20	1.00	.83		
	8	42	20	1.13	.89		
	13	149	-	1.03	-		
	14	129	-	.88	-		
Run No. 18-A							
	1	64	456	11.0	1.05	2/25	65.5;65.9
	2	80	162	11.0	1.05	2/25	67.2;67.4
	3	110	456	11.0	1.05	2/25	67.8
	4	95	162	10.4	1.05	2/25	68.2

	No.	Manometer reading (millimeters)		flask constant		Methionine determination	
		sample	blank	sample	blank	aliquot	% T
Run No. 20							
	1	145	30	1.00	1.00		
	2	191	30	.80	1.00		
	3	172	30	.76	1.00		
	4	158	30	.76	1.00		
	5	148	30	1.01	1.00		
	6	240	4	.80	.97		
	7	146	4	.97	.97		
	8	128	4	1.07	.97		
	9	144	4	.80	.97		
	10	141	4	.84	.97		
Run No. 21							
	2	147	117	.79	1.06		
	3	145	117	.76	1.06		
	4	139	117	.76	1.06		
	5	138	129	1.01	.76		
	6	148	129	.77	.76		
	7	134	129	.95	.76		
Run No. 23							
	2	159	71	.79	1.06		
	3	159	71	.76	1.06		
	4	153	71	.76	1.06		
	5	126	85	1.01	.76		
	6	153	85	.77	.76		
	7	130	85	.95	.76		
Run No. 25							
	1	75	59	1.00	.76	2/5	62.8
	2	leak				2/5	63.9
	3	75	60	.76	.94	2/5	64.0
	4	67	60	.76	.94	2/5	64.9
Run No. 27							
	3	371	169	.76	.81	2/5	70.1
	6	204	126	.76	.83	2/5	67.9
	8	189	126	1.11	.83	2/5	68.4
	9	215	126	.78	.83	2/5	67.9
Run No. 29							
	1	not measured				1/5	61.2
	2	"	"			1/5	60.9
	3	"	"			1/5	62.1
	4	"	"			1/5	60.2
	6	"	"			1/5	62.1

Run No. 19

No.	h' (millimeter)	h (millimeter)	k'_{O_2}	k_{O_2}	k_{CO_2}
1	294		1.15		
7		101		1.11	1.22
4	266		.85		
9		143		.88	.99
5	166		1.09		
10		90		.92	1.03
6	220		.85		
11		125		.93	1.04

Run No. 28

No.	methionine determination		choline determination	
	aliquot	% T	aliquot	% T
1,2	1/10	61.8	1	84.9
3,4	1/10	63.0	1	89.8
6	1/10	62.3		
Blank			1	80.9

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ABSTRACT

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Title of Thesis: An In-Vitro Study of Transmethylation.

Thesis directed by Dr. Fletcher P. Veitch.

Major: Biochemistry

Minors: Physical and Inorganic Chemistry

Pages in Thesis: 124. Words in Abstract: 238.

The reaction between methionine and ethanolamine in the presence of rat liver tissue was studied. du Vigneaud had previously shown by the use of tracer elements that the animal organism is capable of synthesizing choline from dietary methionine and ethanolamine.

It was shown by in-vitro experiments that a simple reaction between ethanolamine and methionine, catalyzed by rat liver tissue, did not occur. The biosynthesis of choline, analyzed by chemical means, could not be demonstrated either under aerobic or anaerobic conditions, with rat liver slices or homogenate. The addition of ATP, DPN, cytochrome-C, and Vitamin B_{12} had no effect on the results. The claim that the disappearance of methionine signified the transfer of methyl groups was disproved. Such disappearance was due solely to the formation of α-keto-γ-methiolbutyric acid.

Possible evidence for the biosynthesis of choline was obtained when choline was determined by the use of choline oxidase in the presence and absence of cobaltous ions. It was also established that the labile methyl group of methionine is oxidized to carbon dioxide

by rat liver tissue in the absence of ethanolamine.

Serine and acetyldimethylethanolamine exhibited no methyl acceptor properties in the biosynthesis of choline. Methyl phosphate showed no methyl donor properties when incubated with ethanolamine and rat liver tissue.

A manometric study in conjunction with a colorimetric determination of methionine presented a convenient method to study the path of the enzymatic deamination of D-methionine. The reaction was shown to be as follows:

$$CH_3SCH_2CH_2CH(NH_2)COOH + \tfrac{1}{2}O_2 \longrightarrow CH_3SCH_2CH_2COCOOH + NH_3$$

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