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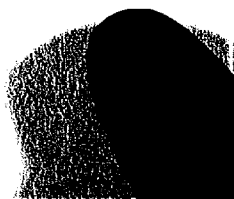
A STUDY OF THE UTILIZATION OF ACETYLMETHIONINE AND ETHIONINE BY THE ANIMAL ORGANISM

by
Laura Winchester Rahm

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A STUDY OF THE UTILIZATION OF ACETYLMETHIONINE AND ETHIONINE BY THE ANIMAL ORGANISM

by Laura Winchester Rahm

The purpose of this work was to study the utilization of certain analogues of methionine, namely N-acetyl-DL-methionine and DL-ethionine, in the animal organism. First, the growth-promoting properties of acetylmethionine were investigated. Young white rats were fed ad libitum a 7.5 per cent casein basal diet and litter mates of these control rats were pair-fed the basal diet supplemented with DL-methionine or N-acetyl-DL-methionine in equimolar amounts. It was found that the protein efficiency (i.e. gain in body weight of the rat per gram of dietary protein consumed) was the same for the two supplemented diets and was much smaller for the unsupplemented basal diet. Acetylmethionine seems to be able to supplement a methionine-deficient (low casein) diet as effectively as methionine. This is interpreted as an indication of a ready cleavage of the acetyl group of acetylmethionine.

The ability of the rabbit to oxidize the sulfur of acetylmethionine was next studied. By determining the sulfur partition (i.e. total, sulfate and organic sulfur) in the urine before and after administration of the compound to the rabbit, it was possible to ascertain the extent of oxidation of the compound in the animal body. It was found that the sulfur of acetylmethionine could be oxidized to sulfate by

the rabbit although not as completely as was the sulfur of methionine. Acetylation of the amino group seems to depress the ease of oxidation of the sulfur in the animal body.

Since the inhibitory effect of ethionine on growth of rats had been determined earlier in other laboratories, its oxidation by the rabbit after oral administration was studied in the present work. It was found that the sulfur of ethionine was not oxidized to sulfate by the rabbit but was excreted as organic sulfur. A large increase in the keto acid excretion was also observed after the administration of ethionine whereas no increase could be detected after the feeding of methionine or acetylmethionine. Ethionine proved to be quite toxic to the rabbit, and, in some cases, fatal.

From the results of this work it would seem that the rat is able to utilize acetylmethionine in place of methionine for growth and that the rabbit can oxidize the sulfur of the acetyl derivative almost as completely as the sulfur of the parent compound. Since the acetylmethionine is more soluble in water than is the methionine, it might substitute for methionine in nutritional and clinical applications.

Ethionine could not be oxidized by the rabbit and appeared to upset metabolic processes: it caused a ten-fold increase in the urinary keto acids and was fatal to several rabbits. The total lipide content of the livers of those rabbits which died was found to be 7-16 per cent of the wet tissue weight, a range which is thought to be high for herbivorous animals.

ACKNOWLEDGEMENTS

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In addition, the help and encouragement of my parents and my husband were essential.

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INTRODUCTION

After the amino acid methionine had been characterized as a constituent of proteins, experiments were undertaken to determine its role in the metabolic processes of the animal organism. It was shown to be utilized by various animals (the sulfur of methionine was oxidized to sulfate) and to serve as a substitute for cystine in the growth of rats. Later studies demonstrated that methionine should be classified as an essential amino acid for the rat and for man.

Various analogues and derivatives of methionine have been studied for physiological properties similar to those of methionine. The promotion of growth of rats on a methionine-deficient diet and the oxidation of the sulfur of the compound by the animal organism have been the two major types of study. The analogues investigated have included compounds similar to methionine in structure with alterations in various groupings in the molecule. Acyl and aryl radicals have replaced the hydrogen atoms of the amino group, the amino group has been replaced *in toto* by an hydroxy or a keto group, the sulfur has been replaced by oxygen or has been oxidized to the sulfoxide or sulfone form, and the methyl group has been removed entirely or replaced with higher homologous radicals. The physiological availability of the analogue depended on the ability of the animal to remove the foreign group or to convert the compound to a metabolite which is normal in the animal organism.

It seemed desirable to study the acetylated derivative of methionine, N-acetyl-DL-methionine. Since the introduction of the acetyl group triples the solubility of the methionine in water, if the acetyl derivative could substitute for the parent compound, the acetylmethionine might be useful in nutrition. Studies with acetylmethionine in this work have included a growth experiment with rats on a methionine-deficient diet and feeding experiments with rabbits to determine the ability of the rabbit to oxidize the sulfur.

Ethionine, the ethyl analogue of methionine which has been of considerable interest for several years, was also studied. Results of earlier experiments seem to indicate that ethionine competes with methionine in the mechanisms by which the methionine is metabolized. Administration of ethionine serves to inhibit the normal processes and to produce toxic effects in the animal. Since other workers had found ethionine to be inhibitory to growth of rats, only the oxidation by rabbits was investigated.

SURVEY OF EARLIER WORK

With the discovery of methionine in various proteins by Mueller (1-3) and Odake (4) over the period 1921-25 and the characterization and synthesis of this amino acid by Barger and Coyne (5) in 1928, many problems regarding the sulfur-containing components of protein were solved. Larger amounts of sulfur were present in protein than could be attributed to cystine, the only sulfur-containing amino acid which was known up to that time; and this "extra" sulfur was firmly bound and not of the lead-blackening variety (labile) as is cystine sulfur. "Methionine" (5) was immediately accepted as the name for α-amino-δ-methylthiolbutyric acid:

$$CH_3\text{-}S\text{-}CH_2CH_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCOOH$$

METHIONINE

Many experiments were then undertaken to determine the physiological availability of this newly-found amino acid and to establish its role in animal metabolism. Mueller (6) ingested a small amount of the crystalline product and found an increased urinary excretion of inorganic sulfate. This indicated that the organic sulfur of methionine was easily oxidized in the body to sulfate and strengthened the idea that the compound might be a primary product of protein digestion. That the increased sulfur was not due to extensive catabolism of the tissues was proved by the fact that no parallel increase in urinary nitrogen was observed.

Subsequent experiments on the dog (7,8) and the rabbit

(9,10) showed that methionine sulfur, administered orally or subcutaneously, was oxidized as readily as cystine sulfur.

In 1933 absorption studies with methionine showed that, in the gastrointestinal tract of the white rat, methionine was as quickly absorbed as was cystine (11) and the formation of sulfate from cysteine and methionine by rat liver and kidney slices in vitro was demonstrated the following year (12). It was also noted that the fluid in which the tissue slices were shaken with methionine yielded a definite nitroprusside reaction* whereas control experiments without methionine gave negative tests. It was then suggested that the methionine lost its methyl group in a demethylation reaction (13). Since the rate of oxidation of methionine was slower than that of cysteine and since cystine could be oxidized only after undergoing reduction to cysteine (12), it would seem that the presence of the free thiol group was necessary for oxidation of the sulfur. This agreed with the idea that demethylation had to precede oxidation of the sulfur in the animal organism (13).

The metabolism of sulfur-containing compounds was also studied in cystinuric individuals. (Cystinuria is an inborn error of metabolism in which large amounts of cystine are excreted in the urine.) It had been found that the feeding of cystine to one of these patients would result in no increase in the urinary cystine whereas cysteine would cause an increase

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* A positive color reaction with sodium nitroprusside indicates the presence of a free sulfhydryl group.

in the cystine in the urine. Ingestion of methionine also resulted in an increased excretion of cystine (14-16). This suggested that methionine was metabolized via cysteine in the body (14,16) and that cystine in cystinuric urine originated in the methionine in the dietary protein (17,18). The level of protein in the diet was found to influence the extent of metabolism of methionine in cystinuria (19,20). On a high protein diet the ingestion of methionine caused little extra cystine in the urine, but the administration of methionine with a low protein diet resulted in a greater increase in urinary cystine.

In addition to these studies of the metabolic relationship and oxidation of methionine in the animal organism, growth experiments with rats were being carried out. Jackson and Block announced in 1931 that methionine would stimulate the growth of rats on a cystine-deficient diet (21,22), and soon thereafter Weichselbaum and coworkers confirmed these results (23). Further work indicated that D-methionine was as effective as the L-isomer in promoting growth (24,25). This was in contrast to the cystine isomers since the D-cystine could not replace the L-cystine on any of the diets tested (26). Since L-cysteine and not D-cysteine could replace L-cystine in the diet (27), it must be assumed that a metabolic conversion of methionine to cysteine (28) and to cystine (28-30) took place through an optically inactive intermediate compound. The L-form of the cysteine or cystine would then be synthesized preferentially in the body.

Various derivatives of methionine were tested for their growth-promoting power in order to gain insight into the metabolism of this very important protein constituent. Homocystine, the next higher homologue of cystine, was synthesized (31-33) and found to be capable of supporting growth of rats on a cystine-deficient diet (32,34). Later, when both the D- and L-isomers of homocystine were prepared (35) and found to be capable of replacing methionine in the diet of the rat (36), the demethylation of methionine with the formation of homocysteine seemed certain. Brand and his coworkers found that in a cystinuric, homocystine was almost completely oxidized whereas half of the extra sulfur excreted after the feeding of homocysteine was as extra cystine (37). They stated that homocysteine was the first major intermediate in the conversion of methionine to cysteine and that the crucial point in the transformation was the maintenance of homocysteine in its reduced state.

The possibility of a specific metabolic relationship of homocystine and methionine to cystine rather than the mere utilization of the sulfur supplied by them was investigated by the preparation and testing of similar substances. Pentocystine (38) and hexocystine (39), the 5- and 6-carbon homologues of cystine, and homomethionine (38) and hexomethionine (39), the next higher homologues of methionine, were found to be incapable of supporting growth of rats on a diet low in cystine. Other experiments showed that S-methylcysteine did not effectively supplement the basal cystine-deficient diet in the rat (40) although it was

readily oxidized in the rabbit (41). The physiological availability of homocystine and methionine is, then, not a general property of amino acids which contain the disulfide or the methyl thiol grouping. The 4-carbon chain seems to be specifically required.

With the aid of synthetic amino acid diets in place of the usual protein diets, Rose and his associates were able to study the ability of cystine and methionine to substitute for each other (42,43). They found that methionine could replace L-cystine completely but L-cystine would not serve in place of methionine in a diet both cystine- and methionine-deficient. It became evident that methionine was the essential amino acid and only one-sixth of the methionine requirement could be satisfied by cystine (44).

Studies with homocystine and homocysteine by Rose and Rice showed that these amino acids could substitute for methionine in a diet of the crystalline amino acids, but the effectiveness of the substitution depended on the nature of other components in the food (45). If a milk concentrate served as the source of vitamin B in the diet, growth was good. If, on the other hand, the pure crystalline vitamins (thiamine, riboflavin and niacin) were used, growth was decidedly at a slower rate. At the same time, duVigneaud and his associates found that neither homocystine nor homocysteine elicited a growth response as good as that obtained with methionine (46). Subsequent experiments indicated that the presence of choline or betaine in the diet might enable the

rat to utilize homocystine for growth purposes in place of methionine; i.e. choline might make possible the in vivo methylation of homocysteine to methionine (47,48).

The Labile Methyl Group

The difficulties involved in separating methionine from tissues made it almost impossible to test the possible transfer of methyl groups from choline to homocysteine, but the reverse process could be established. Methionine labeled with deuterium in the methyl group was fed to rats on a methionine-choline-free diet and the choline and creatine, isolated from the tissues, and the creatinine, isolated from the urine, were found to contain deuterium (49,50). The transfer of the methyl group from methionine to choline and creatine was thus established. Later with the aid of C^{14} and deuterium to label the methyl group of methionine, the ratio $C^{14} : H^{2}$ in the methyl of choline and creatine served to prove that the group was transferred as a unit without exchange of hydrogen atoms (51). It is interesting to note that in 1894 Hofmeister had suggested that the formation of choline and creatine might be due to the same methylating mechanism as the methylation of tellurium (52).

The biosynthesis of methionine was proved by the ingestion of deuteriocholine and homocystine and the detection of the deuteriomethyl group in tissue methionine (53). Since the transmethylation reaction took place when the deuteriocholine was fed without homocystine and even when ordinary

methionine was fed with deuteriocholine, the presence of a methyl acceptor did not seem to be essential for transmethylation. The continued synthesis of methionine, even when it is adequately supplied in the diet, places transmethylation from choline to methionine in the class of "automatic and non-interruptable biochemical processes" (54).

In the experiments which established transmethylation as a physiological process, an occasional animal was able to grow on the homocystine supplement to the basal cystine-deficient diet without any choline or other known methyl donor in the diet. In another laboratory, growth on homocystine without choline was consistently obtained (55-57). It was finally determined that without folic acid and vitamin B_{12}, there would be no methylation of homocysteine to methionine *in vivo* in the rat (58,59) or in the chicken (60). *In vitro* studies with rat liver homogenates showed that the livers of B_{12}- or B_{12}-folic acid-deficient rats had a lowered capacity for synthesis of methionine from homocystine. The addition of B_{12}, folacine, or the Citrovorum Factor to the deficient homogenates did not alter the results (59, 61). Vitamin B_{12} evidently plays a decisive role in the live animal in the formation or utilization of the synthesized labile methyl groups (62). Some work has indicated that B_{12} concentrates added to rat liver slices catalyze the reduction of homocystine to homocysteine (63). Perhaps this is the mechanism by which vitamin B_{12} stimulates the uptake of methyl groups.

Evidence was presented in 1950 that choline is unable to

transfer a methyl group directly to homocysteine for methionine formation (64). In rat liver homogenates incubated with choline and homocysteine, methionine was formed; but the expected dimethylaminoethanol, which would result from demethylation of choline, could not be detected. However, evidence that the demethylation of betaine gave rise to dimethylglycine was obtained (isolation). This would indicate that choline cannot lose a methyl directly but must first be converted to betaine before transfer can occur. This is supported by the results of Borsook and Dubnoff who found that, in rat liver slices, methylation of homocysteine proceeded faster with betaine than it did with choline as the methyl donor (65).

The excretion of methylated compounds by the rat has recently served as a measure of the extent of biosynthesis of methionine on various diets with and without vitamin B_{12} (66). Urinary excretion of N-methyl nicotinamide or of creatinine was determined after an intraperitoneal dose of nicotinamide or of guanidoacetic acid. Only when the basal diet was supplemented with homocystine in the presence of choline, betaine, or vitamin B_{12} was growth equal to that observed on the methionine-supplemented ration. Excretion levels of N-methyl nicotinamide and of creatinine paralleled the weight gains on the various supplements. Vitamin B_{12} is thus proved to be involved in the biosynthesis of methionine.

If sufficient methyl groups to meet the needs of the body are not supplied in the diet, they must be synthesized by the animal organism. At one time it was thought that the

intestinal bacteria might be responsible for this synthesis. This was proved to be erroneous by duVigneaud and his co-workers (67). They fed water containing deuterium oxide to sterile rats and isolated choline from the tissues. The fact that the choline contained deuterium in the methyl groups indicated that biologically labile methyl groups were synthesized.

After it had been determined that the C^{14}-labeled methyl group of methionine was oxidized *in vivo* to C^{14}-labeled carbon dioxide (68), various one-carbon compounds were investigated as possible metabolites of the methyl group. When it was discovered that the methyl of choline could form the ß-carbon of serine (69) and it was recalled that the carbon of formate could be incorporated into serine in the ß-position (70), a close relationship between the methyl group and formate was indicated. It was suggested that the *in vivo* oxidation of methyl to carbon dioxide might occur via formate. With liver homogenates and slices, Mackenzie was able to detect the formation of radioactive formaldehyde, formic acid and carbon dioxide in the oxidation of sarcosine labeled with C^{14} in the methyl group (71,72). In the whole animal, the administration of radiosarcosine resulted in C^{14}-labeled formic acid and formaldehyde in the urine. Since the methyls of methionine and betaine contribute to sarcosine, labile methyl groups are sources of formaldehyde and formate in the body. In a similar experiment radiobetaine administered to the rat produced C^{14}-labeled formic acid in the urine (72).

The experiments mentioned above tested the fate of

methyl groups in the body. Later studies were concerned with the in vivo synthesis of labile methyl groups. Serine labeled with C^{14} in the ß-carbon was injected intraperitoneally in the rat and the choline, isolated from the liver, contained C^{14}-labeled methyl groups (73). Acetone labeled in the methyl group with C^{14} was administered per os to the rat and some of the radioactivity was recovered, in the visceral protein, in the methyl groups of choline and methionine as well as in the ß-carbon of serine (74). It was suggested that acetone is metabolized via a two- and one-carbon cleavage in which formate and acetate are produced (74,75). Since C^{14}-labeled acetate caused no labeling of the ß-carbon of serine, the formate seemed to be the metabolic intermediate between acetone and labile methyl groups.

Welch and Sakami have reported that C^{14}-labeled formate served as a source for the methyl groups of methionine and choline in the intact rat and in rat liver slices (76,77) and duVigneaud and others found that injection of labeled methyl alcohol, formate or formaldehyde resulted in labeled choline in the tissues (78,79). The methanol is not used directly by transmethylation but rather by oxidation and reduction to labile methyl. A confirmation of these reports with additional information came in 1951 from a paper by Arnstein (80). He found that the ß-carbon of L-serine, the ∠-carbon of glycine, methanol and formate are precursors of the choline methyl group. The ß-carbon of D-serine as well as the carboxyl carbon of glycine are not precursors, and the carbon dioxide

formed in the *in vivo* oxidation of these carbons is not converted to choline methyl groups.

N-Substituted Derivatives of Methionine

The study of the ability of various derivatives of methionine to be oxidized by the animal organism began with an investigation of the effect of blocking the amino group with the benzoyl radical. This alteration in the methionine molecule served to depress the oxidation of the sulfur and to cause an increase in the organic sulfur of the urine (9,10). This was the same effect as had been observed with cystine when the amino groups of this amino acid were blocked with the benzoyl radical (81), the phenyluramino group (82,83) or the phenylhydantoin radical (84). The animal organism seems incapable of splitting the foreign groups to free the amino group.

The D- and L-isomers of N-formylmethionine were tested in rats on a cystine-deficient diet and it was found that the N-formyl-L-methionine was equivalent to L- or D-methionine in its growth-promoting power (24,25). The N-formyl-D-methionine was inactive. (These results may be compared with those obtained with similar derivatives of cystine. The sulfur of the acetyl and formyl derivatives of L-cystine was oxidized in the rabbit to about the same extent as was the sulfur of L-cystine itself, but the corresponding D-derivatives were far more resistant to oxidation than was the D-cystine (85).) Until the time of the experiments reported in this thesis,

there had been no record of the effect of acetylmethionine on growth of the rat on a cystine-methionine-deficient diet or of its oxidation in the animal organism. The results here reported show that N-acetyl-DL-methionine is as effective in the promotion of growth in the rat as is the DL-methionine and that the sulfur is oxidized to sulfate in the rabbit.

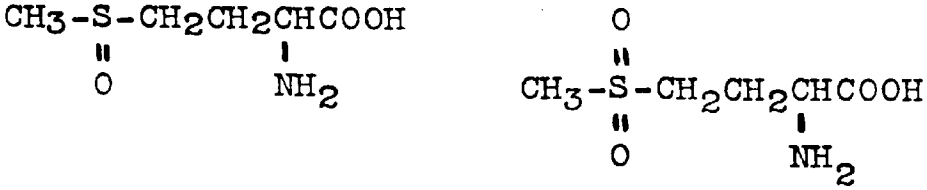
Other Analogues of Methionine

In 1936 Akobe reported results of experiments with α-hydroxy-γ-methiolbutyric acid, the hydroxy acid corresponding to methionine. He found that both the D- and the L-forms were able to promote growth on a methionine-deficient diet (86). The next year Medes stated that the sulfur of the hydroxy analogue of methionine was oxidized completely in the normal human being (87). In the cystinuric, the hydroxy acid was only partially oxidized and much of it was excreted as extra cystine or extra undetermined neutral sulfur (88). (It might be mentioned here that the hydroxy analogue of cystine, α-dihydroxy-β-dithiopropionic acid, was not oxidized in the normal human being (87) and was incapable of replacing cystine for growth in the rat (89).)

After Waelsch and Borek had shown that kidney slices were able to convert methionine into its keto acid, α-keto-γ-methiolbutyric acid (90), it was thought that the reverse process might take place in the body. Cahill and Rudolph found that the keto acid corresponding to methionine was equal to DL-methionine in the promotion of growth and thus

established the utilization of the keto acid in the animal organism (91). It is possible that the first step in the metabolism of methionine is an oxidative deamination rather than a demethylation. In earlier work, duVigneaud had demonstrated that both N,N'-dimethylhomocystine and N-monomethylmethionine were able to support the growth of animals on a cystine-deficient diet and had suggested that the utilization of these methylamino acids proceeded through a deamination to the keto acid (92). The oxidative deamination of methionine followed by demethylation would result in the keto acid corresponding to homocysteine; this sequence of reactions would offer an explanation for the means by which the methylamino acids are utilized in the animal body, i.e. through their keto acids.

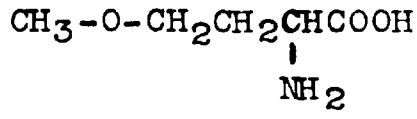
Other derivatives prepared and tested were the oxidized forms of methionine, i.e. DL-methionine sulfoxide and sulfone. The sulfoxide was found to be equal to methionine in growth



METHIONINE SULFOXIDE METHIONINE SULFONE

promotion for the rat (93,94) and for L. arabinosus (95) but the sulfone was ineffective (95-98). Oxidized casein (i.e. casein treated with hydrogen peroxide to convert all the methionine to methionine sulfone) was also found to be incapable of producing growth in rats on a labile methyl-free

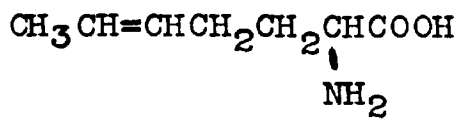
homocystine diet (98). This constitutes indirect proof of the failure to methylate the homocystine and suggests that oxidation to the sulfone destroys the biological lability of the methyl group. The fact that the DL-methionine methyl sulfonium chloride was capable of replacing methionine indicated that the rat was able to metabolize the sulfonium salt (97).



METHOXININE

Methoxinine was found to prevent the growth of E. coli and Staph. aureus and the action could be reversed by L-methionine, though not by D-methionine (99). Thompson has reported that methoxinine inhibited the production of vaccinia virus in the Maitland type of tissue cultures (100) and Shaffer and Critchfield have demonstrated the lipotropic activity and extreme toxicity of methoxinine for rats fed a high fat-low protein diet (101).

The vinylene analogue of methionine has been found to

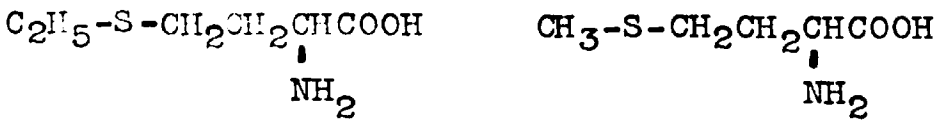


2-AMINO-5-HEPTENOIC ACID

inhibit the growth of one strain of E. coli but the effect was reversed by methionine (102).

Ethionine

Perhaps the greatest amount of experimentation with any one analogue of methionine has been devoted to ethionine (103).



ETHIONINE METHIONINE

Dyer in 1938 was the first to synthesize this ethyl analogue of methionine and to feed it to rats to test its growth-supporting ability on a 5 per cent casein (cystine-deficient) diet (104). She found that not only was the ethionine unavailable for growth but apparently it was quite toxic. Since methionine could partially reverse the growth inhibition, it would seem that the alkylthiol group is specific in the replacement of L-cystine by methionine. Later work showed that ethionine would also inhibit the growth of E. coli due to competition with methionine (105).

In 1948 Hardwick and Winzler confirmed the toxic action of ethionine on growth of rats and the partial reversal with methionine. They also reported a lipotropic effect in the livers of rats ingesting ethionine (106). In these experiments, the basal diet contained 18 percent arachin and hence was low in methionine as well as in choline. During the next year Stekol and Weiss studied the ethionine effect more thoroughly (107). They found that L-, D-, or DL-ethionine would inhibit growth of rats and that either L- or D-methionine would alleviate the inhibition by either isomer of

ethionine. Neither cystine nor homocystine was capable of reversing the growth inhibition but choline was effective. These workers suggested that ethionine might cause an increase in the need for choline and thus divert methionine from other methylation reactions to the synthesis of choline. They found no evidence of fatty infiltration of the liver and concluded that the inhibition of growth by ethionine did not interfere with the lipide turnover in the liver on a diet which was somewhat low in labile methyl groups (107).

In contrast to these results, Tarver and his coworkers have found that a single massive dose of DL-ethionine administered intraperitoneally produced fatty liver in a fasted female rat in 12 hours and death within 24 hours (108,109). The fat accumulation could be prevented by L-, D-, or DL-methionine and by oral doses of glucose or sucrose but was lowered only slightly by choline. It was also reported that the ethionine would not produce fatty liver in males or in castrated males with implanted pellets of testosterone, but was effective only in females and untreated castrated males (109,110).

The same group determined that an injection of ethionine would greatly reduce the incorporation of S^{35}-labeled methionine and of carboxyl-labeled glycine into liver protein in the intact rat and in liver slices (108, 111). Equimolar doses of methionine would reverse this inhibition. Ethionine was also effective in inhibiting the conversion of methionine to cystine *in vivo* but large doses of methionine were required

to relieve the inhibition. It was suggested that the ethionine served to prevent the demethylation of methionine.

It is also possible that the ethionine is incorporated into the tissue protein of the rat. "To upset protein synthesis, the organism might be presented with amino acid analogues capable of being built up in peptide linkage but incapable of proper function in the completed protein" (112).

Further work by duVigneaud and his associates showed that ethionine in the diet would decrease the amount of transmethylation from methionine to choline by about 20 per cent but would not affect the amount of creatine synthesized from dietary methionine (113).

Other deleterious effects of ethionine have been reported by various groups. The acute pancreatitis which is associated with ethionine and which could be entirely prevented by methionine, is believed to be due to some interference with the normal utilization of methionine (114). It was suggested that the inhibition of protein synthesis (due to methionine deficiency) caused the lesion in an organ which is known to be very active in protein synthesis. Another paper the same year reported the degenerative changes in pancreatic acinar tissue within 12 hours after a single injection of ethionine (115). With daily injections, complete obliteration of the acinar tissue was observed. There seemed to be no histological evidence of islet damage.

The structural specificity of ethionine in inducing fatty livers was studied by Tarver (116). It was found that

ethionine sulfoxide was slightly less effective than ethionine in the production of fatty liver and that S-propylhomocysteine, S-isopropylhomocysteine, S-methylcysteine and S-ethylcysteine would not induce fatty liver. This would indicate that both the ethyl group and the aminobutyric radical are required. The protective action of methionine was also found to be structurally specific. S-methylcysteine, the next lower homologue of methionine, would not prevent the rise in liver lipides when injected along with ethionine in amounts equimolar to the ethionine doses. (Methionine was found to protect against fatty liver even when the molar ratio of methionine to ethionine was as low as 1:4.) These findings suggested that the ethyl analogue of methionine induced fatty livers by competing for the enzymes involved in the metabolism of methionine (116).

Stekol and Weiss, with the aid of isotopes, were able to demonstrate the in vivo deethylation of ethionine in the rat (117,118). They injected ethionine with a C^{14} label in the methylene carbon of the ethyl group and found radioactivity in the trimethylamine moiety of choline and in creatine in the tissue. A label of S^{35} in the ethionine which was administered along with p-bromobenzene was recovered in p-bromophenylmercapturic acid in the urine. Degradation of the mercapturic acid showed that the radioactive sulfur was present in the cysteine portion of the molecule. It is evident, therefore, that the ethyl group is available to rats for synthesis of ethyl analogues of normally methylated metabolites

and ethionine sulfur is available to the rat for cysteine synthesis. These results would tend to invalidate the interpretation of Tarver regarding the ethionine inhibition of protein synthesis (111). In the paper reporting this work it was stated that the interpretations would hold only if deethylation did not occur. As a result of this work of Stekol and Weiss, the Tarver experiments do not establish the role of ethionine in inhibiting protein synthesis. The proposal was advanced that the inhibition of growth was probably due to the in vivo synthesis of ethyl analogues of normally methylated metabolites (117,118).

The suggestion of the formation of the ethyl analogues of choline would recall the work by duVigneaud which showed that triethyl choline could not support growth of rats on a homocystine diet and that the diethyl derivative was toxic (119). (It has also been found that the triethyl choline was almost as effective as choline itself in preventing fat deposition in the liver of rats on a high fat-low choline-low protein diet (120) whereas the tripropyl derivative was without action (121).) It is possible that the triethyl choline, like ethionine, causes formation of ethyl analogues of methyl-containing metabolites (122).

Recent experiments by Stekol and Weiss have shown that ethionine would inhibit the growth of weanling mice (C3H strain) on a complete 30 per cent casein diet which contained ample methionine (123). Since the inhibition could be alleviated by giving either choline or additional methionine, but

not by adding cystine or homocystine to the diet, the metabolic disturbance may be concerned with groupings common to methionine and choline, i.e. labile methyl groups. It is interesting to note that no lipide accumulation was observed in the livers of the ethionine-fed mice of either sex.

The work with ethionine which has been discussed thus far has been concerned primarily with growth, protein synthesis and lipide accumulation. A single experiment has been reported which tested the ability of the animal organism to oxidize the sulfur of ethionine. Shen and Lewis in 1946 fed ethionine to a rabbit and noted a great increase in the organic sulfur of the urine with no increase in the sulfate fraction (124). This would indicate that the rabbit is not able to utilize ethionine. Since only the one experiment was performed at that time, the work has currently been repeated in an effort to duplicate their results. The experiments reported in this thesis show that ethionine sulfur is not oxidized by the rabbit and that ethionine is quite toxic. Fatty livers have been observed in those animals which died after ethionine dosage.

EXPERIMENTAL PROCEDURES

OUTLINE OF WORK

The experimental work which is reported here was undertaken in order to study the utilization of certain analogues of methionine in the animal organism. Although acetylmethionine had been synthesized in 1932 by duVigneaud and Meyer (125) and again in 1942 by Kolb and Toennies (126), no studies of its physiological properties have been reported. It was of interest to determine the ability of acetylmethionine to substitute for methionine in the animal organism since the acetylated derivative is several times more soluble in water than the parent substance and hence it might be useful in nutrition.

Experiments were carried out to compare the acetylmethionine with methionine in its ability to promote growth in the young white rat and to be oxidized by the rabbit.

Ethionine, the ethyl analogue of methionine, had also been synthesized earlier (104). The inhibitory effect of ethionine on the growth of young rats had been reported (104) and confirmed (106,107), but in a single experiment only the extent of oxidation of the sulfur of ethionine by the rabbit had been determined (124). Present experiments repeated this study of the administration of ethionine to the rabbit and the analysis of the urine for the sulfur partition and for keto acid excretion.

N-acetyl-DL-methionine

1. Growth experiment with rats.

For the study of the growth-promoting properties of N-acetyl-DL-methionine, young male albino rats were obtained from Maguran Farms in Dixboro, Michigan. The rats, in litter units, were divided into groups of three and were maintained in individual cages equipped with food cups and water bottles. One rat in each litter unit received the 7.5 per cent casein basal diet ad libitum and the food which was consumed each day was measured. A second rat in each litter was fed the basal diet supplemented with 0.6 per cent DL-methionine and was allowed only as much food as the basal rat in that group had consumed on the preceding day. The third rat was also pair-fed with the first, receiving the basal diet supplemented with 0.77 per cent acetylmethionine (equimolar in amount to the methionine supplement). The composition of the diet and the salt mixture are presented in the Appendix. Food which was not consumed or which was spilled was weighed and then discarded to insure freshness of the diet each day. The rats which received the acetylmethionine supplement always consumed all of the food which was offered to them but the methionine group occasionally left some of the diet in the food cup. The rats were weighed frequently during the experimental period of six weeks. In the calculation of results which are presented here, the weight gain represents the final five weeks of the experiment. The first week is thus allowed for adjustment to caging and to the diet.

Results. The data obtained in the growth experiment with acetylmethionine are tabulated in Table I. Values are given for the initial and final weight of each rat, the gain in weight over the experimental period, and the weight of the food consumed by each rat during this period. From these data have been calculated the gain per 100 grams of food and the protein efficiency of the diet (the gain in weight per gram of dietary protein consumed). Average values for the six or seven rats within each diet grouping are also given. The results indicate that acetylmethionine was able to replace methionine for growth in the rat on the low-protein diet. The mean weight gain per 100 grams of food for the rats on the basal diet was 16.8 grams as compared with 24.8 grams for the rats on the methionine supplement and 25.5 grams for the animals receiving supplemental acetylmethionine. The difference between the growth-promoting power of the methionine and the acetylmethionine is insignificant.

2. Experiments with rabbits.

The rabbits used in this work were males weighing between two and three kilograms. They were housed in the usual type of metabolism cages equipped with food and water cups. A constant diet of 50 grams of oats and 80 grams of fresh cabbage was fed throughout the experimental periods and was entirely consumed each day with the exception of a few post-experimental days. The fact that rabbits kept as long as six months on the diet neither lost nor gained weight is

TABLE I

COMPARATIVE GROWTH OF YOUNG RATS FED A BASAL DIET AND SUPPLEMENTARY DL-METHIONINE AND N-ACETYL-DL-METHIONINE

Under the heading Rat no. the first figure indicates the litter unit and the second the number of the rat. Thus rats 1-1, 1-2 and 1-3 were litter mates pair fed with various diets. Results are calculated for a 5-week period.

Standard deviation for the values of gain in weight per 100 gm. food and of gain in weight per gm. protein was calculated by the formula: $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$ (127).

Rat no.	Weight			Food consumption	Gain in weight	
	Initial	Final	Gain		per 100 gm. food	per gm. protein
	gm.	gm.	gm.	gm.	gm.	gm.
				BASAL DIET		
1-1	81.2	140.7	59.5	385.2	15.5	2.06
2-4	68.2	106.9	38.7	286.2	13.5	1.80
3-7	70.1	117.3	47.2	285.6	16.6	2.21
4-10	60.0	103.7	43.7	225.1	19.4	2.58
5-14	80.2	134.5	54.3	340.4	16.0	2.14
6-17	63.3	123.3	60.0	303.3	19.8	2.66
				Average	16.8±1.0	2.24±0.13
				DL-METHIONINE SUPPLEMENT		
1-2	84.8	170.0	85.2	392.6	21.9	2.92
2-5	76.5	138.1	61.6	294.0	20.9	2.79
3-8	78.2	157.2	79.0	291.7	27.2	3.62
4-11	66.0	131.8	65.8	224.0	29.4	3.92
5-15	76.8	143.5	66.7	286.5	23.3	3.11
6-18	71.2	142.4	71.2	276.2	25.8	3.44
				Average	24.8±1.3	3.30±0.18
				N-ACETYL-DL-METHIONINE SUPPLEMENT		
1-3	87.8	165.8	78.0	393.1	19.9	2.66
2-6	76.7	144.8	68.1	294.0	23.2	3.10
3-9	80.0	149.2	69.2	289.4	24.0	3.20
4-12	68.2	133.4	65.2	225.1	29.0	3.87
4-13	68.9	135.5	66.6	225.1	29.6	3.94
5-16	72.6	158.9	86.3	333.9	25.8	3.44
6-19	67.4	149.4	82.0	303.1	27.0	3.60
				Average	25.5±1.3	3.40±0.17

evidence that the food supplied was sufficient for maintenance. The rabbits were allowed on the floor of the laboratory at frequent although brief intervals so that their activity would not be too severely restricted by the small cage.

When a 24-hour sample of urine was to be collected, emptying of the bladder at the beginning and end of the period served to insure the completeness of the specimen. This was accomplished by gentle pressure on the abdominal wall and the urine thus obtained was added to that present in the collecting bottle beneath the metabolism cage. Chloroform was used as a preservative in the collecting bottle. The daily urine was filtered through glass wool and made up to a convenient volume with distilled water. Generally 250 ml. seemed adequate for the analyses performed.

The acetylmethionine was administered in aqueous solution by means of a stomach tube and routine determinations of several urinary constituents were carried out in order to determine the metabolic pathways of the acetylmethionine. The methods used in the determination of sulfur partition, keto acid content and creatinine content are outlined below. A more complete discussion will be found in the Appendix.

The analysis of the urine in the present work involved the establishment of a control level of excretion of the various constitutents over a period of four to six days, the administration by stomach tube of an aqueous solution of the acetylmethionine and the subsequent analysis of the urine for 24-hour periods until the excretion again reached

the control level. Although there was little or no change in the keto acid and creatinine excretion during the experimental period, the sulfur content of the urine was increased. By the third day after administration of the acetylmethionine the sulfur excretion had dropped to the normal level. Subsequent days then served as the control period for the next compound to be administered. In this manner the effect of various compounds on the urinary sulfur, keto acids and creatinine could be studied in the same rabbit.

Sulfur partition. Total sulfur was determined in triplicate by the Benedict-Denis method (128,129) on a volume of the diluted urine equal to approximately one-tenth of the total daily volume. The method involved the oxidation of the sulfur in the urine to the sulfate form by a solution of cupric nitrate, ammonium nitrate and sodium chloride and subsequent precipitation of the sulfate as barium sulfate. Gravimetric determination of the barium sulfate precipitate served to determine the amount of total sulfur in the urine. For analysis of the total sulfate (inorganic plus ethereal sulfate) the method of Folin (130) was used. Duplicate samples, each representing one-fifth of the total urine volume, were satisfactory. The samples of urine were hydrolyzed by heating with hydrochloric acid and the sulfate was then precipitated as barium sulfate. The organic sulfur content of the urine could be determined by subtracting the total sulfate sulfur from the total sulfur value of the sample.

Keto acid excretion. The method for analysis of the keto acid content of the urine was that of Case (131) as modified by Shacter (132) and Knauff (133). This determination involved the reaction of the keto acids in a sample of the urine with 2,4-dinitrophenylhydrazine in acid solution, the extraction of the 2,4-dinitrophenylhydrazones from the aqueous solution by an organic solvent (benzene or ethyl acetate), and the subsequent extraction from the organic solvent layer by a borate buffer with a pH of 8.8. Addition of strong alkali to an aliquot of the buffer extract produced a bright red color which could be measured in the Evelyn photoelectric colorimeter.

It had been found by other workers that benzene would not extract the hydrazones of all keto acids from the aqueous urine solution (133,134). For the extraction of the pyruvic acid hydrazone, benzene was satisfactory, but for the determination of the hydrazone of α-ketoglutaric acid ethyl acetate was far better than benzene. A Japanese investigator suggested the use of xylene to extract the pyruvic acid hydrazone from the urine solution and the subsequent extraction of the aqueous residue by ethyl acetate in order to determine the α-ketoglutaric acid (135). This modification was employed by Knauff who found it quite satisfactory. In the present work, benzene was used as the first organic extractant and ethyl acetate as the second. The results of the keto acid determinations have been tabulated so as to show the keto acids extracted by the benzene and those subsequently extracted by

the ethyl acetate. Total keto acid excretion may be determined by a summation of the two values.

Creatinine excretion. The measure of creatinine served to indicate the completeness of the 24-hour sample which had been collected. The picric acid-sodium hydroxide method of Jaffe was used and the color developed was read in the Evelyn colorimeter. The values of creatinine were employed only as a check and, since they were sufficiently constant for the experimental days, they will not be included here. Most of the rabbits were found to excrete 100-125 mg. of creatinine per 24 hours.

Results. In Tables IV, V, and VII in the Appendix are the detailed results from several of the experiments with rabbits. Sulfur partition values for the control, the experimental and the post-experimental periods are given for all the compounds which were administered to the rabbit. From these values, the distribution of the extra sulfur, believed to be derived from the compound fed, has been calculated. These data have been summarized in Table II. The per cent of the sulfur administered as acetylmethionine which was recovered as "extra sulfur" in the experimental urines is given in the third column. Also tabulated are the values for the per cent of the total extra sulfur which is in the total sulfate fraction and the per cent of the total extra sulfur appearing in the organic sulfur fraction.

The partition of the extra sulfur following methionine administration is about 80 per cent in the sulfate fraction and 20 per cent in the organic sulfur portion of the urine.

TABLE II

URINARY SULFUR PARTITION IN THE RABBIT AFTER ADMINISTRATION OF ACETYLMETHIONINE, METHIONINE, AND ETHIONINE

Summary of Results Given in Tables IV-VIII.

Rabbit no.	Compound administered	as sulfur	Total extra sulfur of sulfur administered	Total extra sulfur of total sulfur excreted: as sulfate sulfur	Total extra sulfur of total sulfur excreted: as organic sulfur
		mg.	per cent	per cent	per cent
1	Acetylmethionine	215	64.0	49.7	50.3
1	"	"	85.8	47.2	52.8
1	"	"	88.4	44.5	55.3
2	"	"	92.9	56.5	43.5
1	Methionine	215	88.5	87.4	12.5
2	"	143	68.5	74.4	25.6
1	"	215	80.1	65.5	34.5
2	"	143	82.6	87.0	12.9
2	Ethionine	143	10.4	≠	179.2
2	"	143	95.2	21.8	78.2
2	"	143	68.1*	25.9	74.1
1	"	143	44.5*	15.2	84.7
3	"	98	58.8*	4.5	95.5
3	"	120	59.1*	8.3	91.7

≠ The amount of sulfur present as sulfate was less than the control value.

* These values represent only the first day after administration of the ethionine since the second day showed a decrease in excretion of the sulfate fraction compared with the control days. All other values represent the first two days after dosage. By the third post-dosage day, the excretion had, with one exception, returned to the control level. This one exception showed that more than 100 per cent of the sulfur administered in acetylmethionine was excreted as extra sulfur in the three days following dosage. The value as listed in the table is 85.8 per cent and represents the two day post-dosage period.

This is the distribution of sulfur in the normal urine. The administration of acetylmethionine resulted in a somewhat different sulfur partition. The sulfur of acetylmethionine is partitioned about evenly between the sulfate and organic sulfur fractions. This would indicate that the rabbit was not able to oxidize acetylmethionine as completely as methionine since a larger portion of the acetylmethionine sulfur was excreted in the unoxidized form. No toxic effects of acetylmethionine in the rabbit were observed.

Data for the keto acid excretion from day to day are given in Tables V and VII in the Appendix. The results, summarized in Table III, indicate that administration of neither methionine nor acetylmethionine caused an increase in the keto acids of the urine. It is, therefore, concluded that the rabbit excreted little of the unchanged methionine or acetylmethionine or was able to metabolize these two compounds beyond the keto acid stage.

DL-ethionine.

1. Experiments with rabbits.

The study of the utilization of ethionine in the animal organism involved the analysis of the urine of a rabbit which was fed ethionine by stomach tube. The sulfur partition, the keto acid content and the creatinine content of the rabbit urine were determined, and the methods used were the same as those which have been described for the work with acetylmethionine.

TABLE III

KETO ACID EXCRETION IN THE RABBIT AFTER ADMINISTRATION OF ACETYLMETHIONINE, METHIONINE AND ETHIONINE

Summary of Results Given in Tables V, VII and VIII.

In the tabulation of the results for each compound administered, the first horizontal row represents the control level, an average of the keto acid values for 4 to 6 days preceding the administration of the compound. The second row gives the value for the experimental period and the third row shows the post-experimental excretion. Each of the last two periods represents 24 hours.

Under the heading Benzene are tabulated the keto acid values as determined by benzene extraction of the aqueous urine solution. Subsequent extraction of the aqueous layer with ethyl acetate resulted in the values given under the heading Ethyl acetate.

Rabbit no.	Compound administered	as sulfur	Keto acids excreted		Extra keto acids during experimental period		
			Benzene	Ethyl acetate	Benzene	Ethyl acetate	Total
		mg.	mg./24 hr.	mg./24 hr.	mg./24 hr.	mg./24 hr.	mg./24 hr.
1	Acetylmethionine	215	4.3	3.0			
			6.8	3.8	2	1	3
			3.1	3.7			
2	Acetylmethionine	215	4.4	3.4			
			4.9	3.9	0	0	0
			5.1	4.2			
1	Methionine	215	3.5	2.5			
			10.8	6.0	7	4	11
			6.4	3.5			
2	Methionine	143	4.0	2.8			
			5.8	4.2	2	1	3
			4.0	3.8			
2	Ethionine	143	4.2	3.0			
			54.8	26.2	51	23	74
			8.2	6.2			
1	Ethionine	143	3.6	2.9			
			33.8	16.5	30	14	44
			8.6	8.2			
3	Ethionine	98	7.6	3.2			
			58.3	37.5	51	34	85
			12.6	6.8			
3	Ethionine	120	4.0	3.0			
			48.0	25.0	44	22	66
			7.0	4.6			

Ethionine was found to be quite toxic in the doses administered and was fatal in several cases. Because of this toxicity the amounts of ethionine fed were not equimolar with those of methionine and acetylmethionine in most cases. In the instances of fatality, deaths occurred from 60 hours to one week after the ethionine was administered. The livers of these animals were removed as promptly as possible after the death of the rabbit and were analyzed for lipide content. It was thought that a massive dose of ethionine might cause a fatty liver in the rabbit just as it did in the rat (109,110). For this determination an extraction method was used as described subsequently.

Liver lipide. After the liver had been excised, it was minced, weighed and placed in absolute alcohol. It was allowed to stand in the alcohol for several days and then the liquid was decanted and saved. The liver was extracted with several portions of boiling alcohol and subsequently by boiling ether. All of these alcohol and ether extracts were combined and the solvents were evaporated as completely as possible. The lipide residue was taken up by alternate extractions with petroleum ether and aqueous alcohol and the two solvents were separated in a separatory funnel. The alcohol layer was discarded but the petroleum ether layer was drawn off into a weighed flask and evaporated to dryness. The weight of lipide could be determined by difference in weight of the flask with and without the lipide residue.

Results. A summary of the results of the sulfur partition determinations is given in Table II and the complete data are tabulated in Tables V - VIII in the Appendix. It was found that the major portion of the ethionine sulfur was excreted as extra organic sulfur. About 85 per cent of the extra sulfur was present in the organic sulfur fraction with only 15 per cent appearing in the sulfate portion. These results confirm the single experiment of Shen and Lewis (124), and show that the rabbit is not able to oxidize the sulfur of ethionine to sulfate sulfur.

The results of the keto acid determinations are given in Tables V, VII and VIII in the Appendix and are summarized in Table III. They show that when ethionine was fed to the rabbit, a ten-fold increase in the keto acid excretion was detected. The extra keto acids included those whose hydrazones were extractable by benzene as well as those determined in the ethyl acetate extract. These keto acids were not identified and characterization is necessary before the effect of ethionine in the animal can be explained satisfactorily.

The livers of the rabbits which died after ethionine administration were found to contain more fat than is considered normal for the rabbit. Results are given in Table X. The herbivorous animal would be expected to have a very low content of liver fat (2.3 per cent has been recorded in the literature (136)) and these livers contained 7 to 16 per cent fat. Pathological examinations of the liver tissue indicated that

the livers were cirrhotic as well as fatty.*

2. Chemical studies.

Reaction with nitrous acid. Since it was known that methionine sulfur was not oxidized to sulfate by nitrous acid as was cystine sulfur (137), it was of interest to study the stability of ethionine sulfur with this acid. A sample of ethionine (0.254 gm.) was dissolved in water and solutions of hydrochloric acid (30 ml. of 3.25 per cent HCl) and of barium nitrite (30 ml. of 5 per cent solution, i.e. 1.62 gm. of crystalline $Ba(NO_2)_2$) were added. The solution was allowed to stand for 48 to 72 hours and additional barium nitrite was added at intervals. At no time during this period did a precipitate of barium sulfate appear. It was therefore assumed that nitrous acid did not oxidize the sulfur of ethionine to sulfate sulfur.

The primary reaction of nitrous acid on an amino acid is the replacement of the amino group by an hydroxy group. If the hydroxy acid were then oxidized, the keto acid might result. It was thought that the keto acid derivative of ethionine might be formed by the action of nitrous acid and attempts were made to verify this assumption. To the reaction mixture of ethionine, hydrochloric acid and sodium nitrite was added a solution of 2,4-dinitrophenylhydrazine in hydrochloric acid. The solution was allowed to stand a few minutes and then sodium hydroxide was added until a reddish-brown color developed. This color indicated the

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* Appreciation is expressed to Dr. Carl V. Weller, Professor of Pathology at the University of Michigan, for the histological examination of the liver tissue.

presence of a keto acid in the reaction mixture.

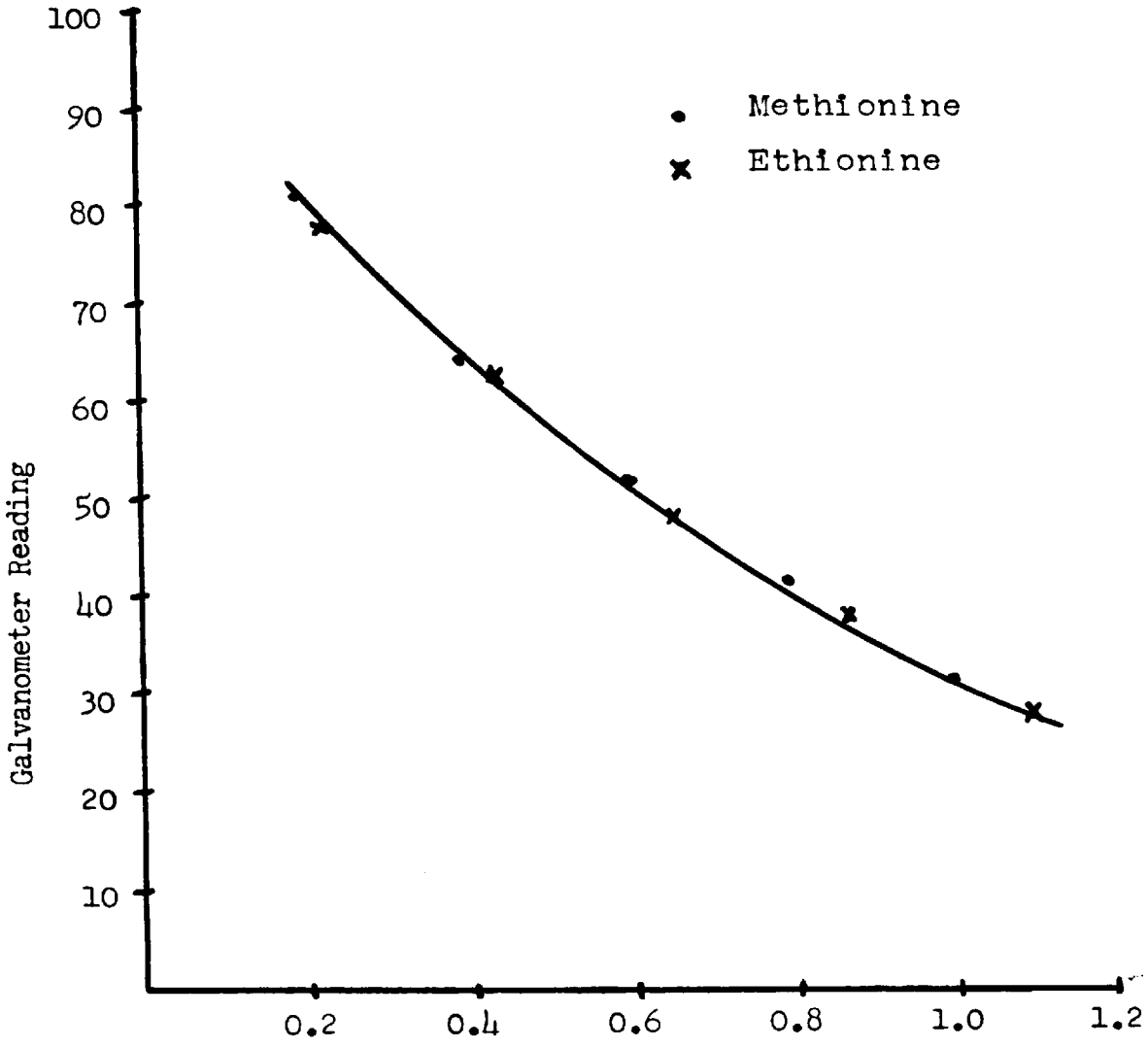
It was desirable to isolate the keto acid as its 2,4-dinitrophenylhydrazone from the solution. A fresh sample of ethionine was mixed with the hydrochloric acid and sodium nitrite as before but with a minimum amount of solvent present. When a saturated solution of 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid was added, a cloudiness developed. The mixture was allowed to stand in the refrigerator overnight. The yellow crystals which separated out on standing were dried and recrystallized. The melting point was found to be 67° C. (uncorrected). Since very small amounts of the crystalline material were obtained in these studies, an elemental analysis by macro methods was impossible and it did not seem advisable to have the sample analyzed by micro methods. If the melting point is a guide to the possible identity of the hydrazone, the melting point of the hydrazone of the keto acid of methionine would be suggestive. Waelsch and Miller stated that the melting point of the methionine derivative was 149° C. (138). It would seem that the corresponding ethionine derivative would have a melting point closer to the 149° value than the 67° observed. Further studies of the reaction of ethionine with nitrous acid might result in larger yields of the hydrazone which might then be identified. These were not attempted.

McCarthy-Sullivan test for methionine. At one stage in the experimental work, it was thought that the McCarthy-Sullivan reaction (139), which can be used as a quantitative test for

methionine, might serve as a means of detecting methionine and/or ethionine in the urine of the rabbit. (It has been reported that ethionine gives a more intense color than an equimolar amount of methionine in this reaction (140).) This test involves the addition of a sodium nitroprusside solution to an alkaline solution of the methionine, and the subsequent development of a red color by the addition of a mixture of hydrochloric acid and phosphoric acid. The measurement of the intensity of the red color can be used as a means of determining quantitatively the methionine content of a solution if a standard curve has been established.

It was found that equimolar amounts of methionine and ethionine gave almost the same intensity of color and the standard curves for the two compounds in this reaction were practically identical (Figure 1). The reaction might then be used to measure the ethionine content of a solution. Samples of rabbit urine collected before and after administration of ethionine were subjected to the reaction. The experimental urine showed a definite increase in "ethionine-like" substances over the control urines. That these color-producing substances were not extracted from the urine by benzene or by ethyl acetate was demonstrated by carrying out the reaction on a sample of urine previously extracted with one of the organic solvents. Almost all of the color produced in a sample of urine was still in the aqueous residue after extraction by an organic solvent. Samples of a standard solution of ethionine were extracted by these same organic

FIGURE 1



Standard Curve for the Quantitative Determination of Methionine and Ethionine by the McCarthy-Sullivan Reaction

(Data for the curve are given in Table XI in the Appendix.)

solvents, and all of the amino acid could be accounted for in the aqueous residue. From this limited amount of work it would seem that some ethionine fed to the rabbit might be excreted unchanged in the urine.

It would be interesting to know whether or not the keto acid derivatives of methionine and ethionine would give this reaction. If the keto acids do give a positive test, perhaps the "ethionine-like" substances in the urine could be sulfur-containing keto acids which were excreted after administration of ethionine. Extraction of the acidified urine with an organic solvent such as benzene or ethyl acetate might be expected to remove keto acids from the aqueous solution. Since the amount of color-producing substances lost by extraction was negligible, the major portion of the urinary keto acids can not be attributed to the derivatives of methionine and ethionine if it is assumed that the sulfur-containing keto acids do give a positive McCarthy-Sullivan reaction.

DISCUSSION OF RESULTS

The work with acetylmethionine was undertaken with the expectation that the compound would not differ greatly in its utilization in the body from methionine itself. Since acetylation reactions of sulfur-containing amino acids take place in the body (synthesis of mercapturic acids) it seemed reasonable to assume that the process might be reversed and that the acetyl group could be removed. The methionine thus formed from the deacetylation of acetylmethionine could then be used in the body. The rate at which the methionine is freed might influence the extent of utilization of the acetylmethionine in the body.

The ability of the N-acetyl-DL-methionine to promote growth of rats on a methionine-deficient diet was not unexpected, but in the light of work in other laboratories it is surprising that equimolar supplements of DL-methionine and its acetyl derivative stimulated growth to the same extent. Jackson and Block reported in 1933 that the N-formyl derivative of L-methionine was able to replace methionine but that the N-formyl-D-methionine could not be utilized by the animal organism (24,25). Later, in 1936, duVigneaud and Dyer stated that they had unpublished results which demonstrated that acetyl-L-cystine was as well used for growth purposes when injected intraperitoneally into rats as when it was fed (141). No report of a comparison with L-cystine in growth-promoting properties was made but it was concluded that the tissues were able to remove the acetyl group. The

next year these same workers reported studies with N-acetyl-homocystine in which only the L-isomer was found to be effective in replacing homocystine for growth (142). The acetyl-D-homocystine could not be utilized by the rat for growth purposes. In the present experiments racemic preparations of DL-methionine and of N-acetyl-DL-methionine were used and were found to be equally effective in promoting growth. It might be assumed, therefore, that the acetyl-D-methionine would not be inferior to the D-methionine in the promotion of growth in the white rat.

The oxidation by the rabbit of the sulfur in acetylmethionine was also expected and the fact that methionine sulfur is oxidized more completely than is the sulfur of acetylmethionine is in line with work reported from other laboratories. Hele and Pirie in 1931 found that the dog could oxidize L- and DL-cystine equally well but that acetylcystine and glycylcystine were not so readily oxidized (143). In the work of duVigneaud and Dyer with N-acetylhomocystine, the oxidation of sulfur of the D-homocystine was found to be decreased by the presence of the acetyl radical (142). The L-homocystine and its acetyl derivative were oxidized to the same extent. Here the acetyl-D-methionine may be oxidized less readily than the D-methionine.

No increase in keto acid excretion resulted after administration of either methionine or acetylmethionine. This would indicate that there was no accumulation of keto acids as intermediate metabolites. The rabbit might have excreted

the compound unchanged (although it could not be detected by the McCarthy-Sullivan reaction) or he might have oxidized it beyond the keto acid stage.

The experiments in which ethionine was administered to the rabbit showed the same results as have been reported by Shen and Lewis (124). The extra urinary sulfur which appeared after administration of ethionine was in the organic sulfur fraction and only negligible increases in total sulfate sulfur were observed. It is evident that the rabbit is unable to oxidize the sulfur of ethionine.

The large increase in keto acid excretion which was reported earlier to follow ethionine administration was also observed. The identification of these keto acids was not accomplished and is necessary before the metabolic pathways of ethionine can be determined exactly. It is possible that the ethionine might have been oxidatively deaminated to its keto acid which could not be handled and was therefore excreted. It is also possible that the keto acids are not derived from the ethionine itself but are the result of a specific stimulation of other metabolic processes (138). The fact that the hydrazones of the keto acids were not all extracted by the benzene might indicate the multiple nature of the keto acids excreted. This would argue for a disturbance of metabolic processes rather than the production and then excretion of the keto acid of ethionine. A valid interpretation of the results must await characterization of the keto acids in the urine.

The lipide contents of the fatty livers which resulted from ethionine dosage were in keeping with the results reported earlier with rats. Tarver had found that a massive dose of ethionine would result in a fatty infiltration of the livers of fasted female rats but not of males (109,110). There would seem to be some species difference here in that fatty liver was produced in male rabbits. There is some indication that the extent of fat accumulation in the liver is influenced by the size of the dose and by the weight of the rabbit. It is evident that insufficient data have been recorded here and elsewhere in the literature for valid conclusions to be drawn.

SUMMARY OF RESULTS

1. N-acetyl-DL-methionine was found to be as effective as DL-methionine in promoting the growth of white rats on a methionine-deficient diet.

2. The sulfur of N-acetyl-DL-methionine was oxidized to sulfate by the rabbit, but not as completely as was the sulfur of DL-methionine. No increase in keto acid excretion was observed after administration of methionine or acetylmethionine to the rabbit.

3. DL-ethionine did not appear to be utilized by the rabbit. The sulfur was not oxidized to sulfate but was excreted in the organic sulfur fraction of the urine. A large increase in keto acid excretion was caused by ethionine administration.

4. Large doses of ethionine were toxic to the rabbit and evidence from a limited number of experiments seemed to indicate that ethionine caused a fatty infiltration of the liver of the rabbit.

APPENDIX

SOURCE AND PURITY OF THE CHEMICALS

USED IN THE EXPERIMENTAL WORK

1. N-acetyl-DL-methionine was supplied by the Chemo Puro Manufacturing Corporation*. Data accompanying the sample were as follows:

   Melting point - 114-116° C.

   Nitrogen - 7.1 - 7.5 per cent. (Theoretical 7.328 per cent).
   Sulfur - 16.15-17.0 per cent. (Theoretical 16.764 per cent).

   Solubility :

Temperature °C.	Grams acetylmethionine/100 ml. saturated solution
20°	8.70
30°	11.60
40°	18.64

   Data obtained in this laboratory were as follows:

   Sulfur (Parr bomb) - 16.67 per cent. (Theoretical 16.76 per cent).

   Molecular weight (acid equivalence) - 193.5. (Theoretical 191.2).

2. DL-methionine was obtained from Winthrop-Stearns Inc.

   Data which have been recorded for the solubility of DL-methionine in water are as follows: (144)

Temperature °C.	Grams methionine/100 gm. water
20°	2.995
30°	3.812
40°	4.824

3. DL-ethionine was purchased from U. S. Industrial Chemicals. It was a portion of Lot B-534.
   Data obtained in this laboratory were as follows:

   Nitrogen (Kjeldahl) - 8.50 per cent (Theoretical 8.58 per cent).
   Sulfur (Parr bomb) - 19.12 per cent (Theoretical 19.64 per cent).

* We are grateful to Mr. P. C. Hereld of the Chemo Puro Manufacturing Corporation for the gift of the acetylmethionine.

BASAL DIET FOR RATS (145)

	per cent
Casein (Labco vitamin free- Borden)	7.5
Sucrose	41.4
Glucose monohydrate	21.1
Cellulose (Ruffex)	5.0
Salt mixture (modified Osborne-Mendel)	4.0
Vitamin mixture (in starch)	1.0
Corn oil (Mazola)	17.0
Cod liver oil	3.0

Dried liver extract (Lilly) 100 mg. fed to each rat daily.

Supplements of methionine and acetylmethionine replaced an equal weight of glucose monohydrate.

Vitamin Mixture

	mg.
Thiamine hydrochloride	37
Pyridoxine hydrochloride	37
Riboflavin	75
Calcium pantothenate	300
Para-aminobenzoic acid	300
Nicotinic acid	375

These were mixed with sufficient starch to give 100 gm. of vitamin mixture.

MODIFIED OSBORNE-MENDEL SALT MIXTURE (145)

	gm.
$CaCO_3$	134.8
$MgCO_3$ or	24.2
$MgCO_3$-$Mg(OH)_2$-$3H_2O$	25.6
Na_2CO_3	34.2
K_2CO_3	141.3
Citric acid	111.1
Ferric citrate-1 1/2 H_2O	6.34
KI	0.020
$MnSO_4$	0.079
NaF	0.248
$KAl(SO_4)_2$ or	0.0245
$KAl(SO_4)_2$-$12H_2O$	0.054
H_2SO_4	9.2 or 5.2 ml. 96% H_2SO_4
H_3PO_4	103.2 or 71 ml. 85% H_3PO_4
HCl	53.4 or 121 ml. 35% HCl
$CuSO_4$	2 ml. of 10% solution

TABLE IV

URINARY SULFUR PARTITION OF RABBIT NO. 1 DURING CONTROL PERIODS AND AFTER ADMINISTRATION OF ACETYLMETHIONINE AND METHIONINE

Day	Sulfur			Extra Sulfur			Compound fed	
	Total	Sulfate	Organic	Total	As sulfate	As organic		
	mg.	mg.	mg.	mg.	mg.	mg.		gm.
1-2	141.3	105.0	36.3					
3-4	113.5	78.1	35.4					
5	139.1	97.6	41.5					
6	151.1	114.6	36.5					
7	246.4	166.6	79.8	110.2	67.8	42.4	Acetylmethionine	1.28*
8	163.7	99.4	64.3	27.5	0.6	26.9		
9	146.6	89.7	56.9	10.4	-9.1	19.5		
10-11	115.3	75.9	39.4					
12-13	122.3	81.0	41.3					
14-15	109.2	76.2	33.0					
16	269.2	211.8	57.4	153.6	134.1	19.5	Methionine	1.0*
17	152.6	110.3	42.3	37.0	32.6	4.4		
18	138.2	103.4	34.8	22.6	25.7	-3.1		
19-20	126.6	90.8	35.8					
21-22	113.2	82.0	31.2					
23	255.9	162.7	93.2	136.0	76.3	59.7	Acetylmethionine	1.28*
24	168.3	97.2	71.1	48.4	10.8	37.6		
25	160.2	103.2	57.0	40.3	16.8	23.5		

* The sulfur content of all compounds fed was uniform, 215 mg. of sulfur.

TABLE V

URINARY SULFUR PARTITION AND KETO ACID EXCRETION OF RABBIT NO. 1 DURING CONTROL PERIODS AND AFTER ADMINISTRATION OF ACETYLMETHIONINE, METHIONINE AND ETHIONINE

Day	Sulfur						Keto acids				Compound fed
	Total	Sulfate	Organic	Extra sulfur			Benzene	Ethyl Acetate	Extra		
				Total	As sulfate	As organic			Benzene	Ethyl Acetate	
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	gm.
1-2	81.7	54.6	27.1				4.65				
3-4	93.5	67.6	25.9				3.42				
5	89.7	65.7	24.0				5.46	2.94			
6	100.7	72.6	28.1				4.22				
7	243.7	131.4	112.3	153.6	67.6	85.9	6.85	3.75	2.55	0.81	Acetyl-methionine 1.28*
8	126.5	80.9	45.6	36.4	17.1	19.2	3.12	3.68			
9	91.9	63.6	28.3	1.8	-0.2	1.9	3.48	3.30			
10-11	95.4	67.5	27.9				2.78	2.02			
12-13	90.9	65.9	25.0				4.00	2.78			
14-15	107.8	79.4	28.4				3.84	2.78			
16	207.7	140.4	67.3	109.7	69.5	40.2	10.85	6.05	7.26	3.52	Methionine 1.0*
17	160.6	114.2	46.4	62.6	43.3	19.3	6.38	3.53			
18	106.4	76.5	29.9	8.4	5.6	2.8	3.58	2.90			
19-20	93.6	67.2	26.4				3.66	2.72			
21-22	91.0	67.4	23.6				3.52	3.08			
23	156.0	77.0	79.0	63.7	9.7	54.0	33.8	16.5	30.2	13.6	Ethionine 0.73#
24	117.7	64.2	53.5	25.4	-3.1	28.5	8.61	8.19			
25	65.4	32.9	32.5	-26.9	-34.4	7.5	5.28	3.12			
26-27	24.0	13.1	10.9								
28	Rabbit died - 7.4 per cent fat in liver.										

* The sulfur content of the acetylmethionine and methionine fed was uniform - 215 mg. of sulfur.

\# The sulfur content of the ethionine fed was 143 mg. of sulfur.

TABLE VI

URINARY SULFUR PARTITION OF RABBIT NO. 2 DURING CONTROL PERIODS AND AFTER ADMINISTRATION OF ETHIONINE AND METHIONINE

Day	Sulfur						Compound fed	
	Total	Sulfate	Organic	Extra sulfur				
				Total	As sulfate	As organic		
	mg.	mg.	mg.	mg.	mg.	mg.		gm.
1-2	130.2	94.8	35.4					
3-4	120.4	81.8	38.6					
5	132.3	95.2	37.1					
6	151.0	116.2	34.8					
7	141.3	89.0	52.3	7.8	-8.0	15.8	Ethionine	0.73*
8	140.6	93.2	47.4	7.1	-3.8	10.9		
9	-	-	-					
10-11	128.7	84.2	44.5					
12-13	132.5	88.7	43.8					
14-15	112.3	77.8	34.5					
16	179.2	122.5	56.7	54.7	38.9	15.8	Methionine	0.67*
17	167.8	117.6	50.2	43.3	34.0	9.3		
18	152.5	115.7	36.8	28.0	32.1	-4.1		
19-20	127.7	90.2	37.5					
21-22	125.6	87.2	38.4					
23	216.2	109.4	106.8	89.5	20.7	68.8	Ethionine	0.73*
24	173.5	97.8	75.7	46.8	9.1	37.7		
25	112.4	63.4	49.0	-14.3	-25.3	11.0		

*The sulfur content of the compounds fed was uniform - 143 mg. of sulfur.

TABLE VII

URINARY SULFUR PARTITION AND KETO ACID EXCRETION OF RABBIT NO. 2 DURING CONTROL PERIODS AND AFTER ADMINISTRATION OF ETHIONINE, METHIONINE AND ACETYLMETHIONINE

Day	Sulfur						Keto acids				Compound fed
	Total	Sulfate	Organic	Extra sulfur			Benzene	Ethyl acetate	Extra		
				Total	As sulfate	As organic			Benzene	Ethyl acetate	
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	gm.
1-2	91.3	71.3	20.0				4.32				
3-4	101.1	74.0	27.1				3.95				
5	84.4	63.0	21.4				4.20	3.00			
6	89.3	68.3	21.0				4.41				
7	190.5	95.5	95.0	97.4	25.2	72.2	54.8	26.2	50.6	23.2	Ethionine 0.73*
8	117.4	62.0	55.4	24.3	-8.3	32.6	8.20	6.20			
9	84.3	47.6	36.7	-8.8	-22.7	13.9	9.98	6.48			
10-11	73.1	25.5	47.6				7.78	3.76			
12-13	58.7	35.6	23.1				4.32	3.8[illegible]			
14-15	100.5	74.1	26.4				3.68	2.94			
16-17	118.0	93.4	24.6				5.62	4.70			
18-19	102.7	82.5	20.2				4.00	2.90			
20	73.5	56.3	17.2				3.95	2.78			
21	224.7	187.8	36.9	114.3	99.8	14.5	5.82	4.22	1.84	1.38	Methionine 0.67*
22	114.4	91.2	23.2	4.0	3.2	0.8	4.02	3.85			
23	104.9	83.1	21.8	-5.5	-4.9	-0.6	4.32	3.85			
24-25	93.3	70.8	22.5				4.80	3.45			
26-27	107.0	84.7	22.3				4.08	3.45			
28	267.7	178.8	88.9	167.5	101.0	66.5	4.86	3.88	0.42	0.43	Acetyl- 1.28# methionine
29	132.5	89.6	42.9	32.3	11.8	20.5	5.19	4.15			
30	102.2	77.9	24.3	2.0	0.1	1.9	4.12	3.12			
31-32	99.9	78.4	21.5				4.32	3.52			

Died on a later dose of ethionine 14.1 per cent fat in liver

* Sulfur content of ethionine and methionine was uniform, 143 mg. of sulfur.

\# Sulfur content of acetylmethionine was 215 mg. of sulfur.

TABLE VIII

URINARY SULFUR PARTITION AND KETO ACID EXCRETION OF RABBIT NO. 3 DURING CONTROL PERIODS AND AFTER ADMINISTRATION OF ETHIONINE

Day	Sulfur						Keto acids				Compound fed
	Total	Sulfate	Organic	Extra sulfur			Benzene	Ethyl acetate	Extra		
				Total	As sulfate	As organic			Benzene	Ethyl acetate	
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	gm.
1-2	107.7	85.4	22.3				6.98	3.25			
3-4	108.5	87.8	20.7				10.70	3.22			
5	105.4	82.2	23.2				5.16	3.08			
6	94.4	74.2	20.2				5.26	3.22			
7	163.0	86.4	76.6	57.6	2.6	55.0	58.3	37.5	50.7	34.3	Ethionine 0.5*
8	118.8	80.3	38.5	13.4	-3.5	16.9	12.6	6.81			
9	102.3	63.4	38.9	-3.1	-20.4	17.3	7.10	4.60			
10-11	128.8	92.3	36.5				12.1	4.42			
1-2	118.0	92.8	25.2				4.21	2.71			
3-4	110.8	87.4	23.4				3.70	3.00			
5	185.3	96.0	89.3	70.9	5.9	65.0	48.0	25.0	44.0	22.1	Ethionine 0.61#
6	134.5	85.8	48.7	20.1	-4.3	24.4	7.02	4.65			
7	102.1	61.6	40.5	-12.3	-28.5	16.2	5.10	4.60			

* Sulfur content of ethionine fed was 98 mg. of sulfur.
\# Sulfur content of ethionine fed was 120 mg. of sulfur.

DETAILED EXPERIMENTAL PROCEDURES

Determination of Total Sulfur in Urine

Reagents.

Denis reagent. Cupric nitrate 25 gm.
Sodium chloride 25 gm.
Ammonium nitrate 10 gm.
Water to make 100 ml.

Hydrochloric acid. A 1:4 solution was prepared by mixing 100 ml. of concentrated hydrochloric acid with 400 ml. water.

Barium chloride. 10 per cent solution.

Procedure.

The method of Benedict-Denis (128,129) was used for the estimation of the total sulfur content of the urine of the rabbit. Triplicate samples of the diluted urine, each representing about one-tenth of the total daily volume, were pipetted into evaporating dishes about 4 inches in diameter, and a portion of five ml. of the Denis Reagent was added to each one. The dishes were placed on the steam bath until the solution had evaporated to dryness and were then carefully heated on a hot plate, through stages of low and high heats, until the contents had fused and solidified into a black mass. The dishes were then placed in the flame of a Bunsen burner for a few minutes and finally ignited over a Meker-Fisher burner for ten minutes. This gradual increase in heat was necessary to prevent spattering. The dishes were allowed to cool before the residue was dissolved in 20 ml. of a 1:4 solution of hydrochloric acid. The bright, clear blue solution was filtered through ordinary filter paper into a 250 ml.

beaker and washed through with 100 ml. of water. This filtration was advisable since it served to remove any particles of silica. A small amount of alcohol (about one ml.) was added to the solution in the beaker and the solution was then heated to boiling. A ten ml. portion of a 10 per cent solution of barium chloride was then added, drop by drop, to the hot solution. The flow from the pipette was controlled by a capillary tube so that it required slightly more than three minutes for the 10 ml. to drop into the solution. The mixture was allowed to stand undisturbed for an hour. At the end of this time the mixture was thoroughly stirred and filtered, with the aid of suction, through a Gooch crucible which had been previously brought to constant weight. The precipitate was washed with 100 ml. of water followed by a few ml. of alcohol, first rinsing the beaker with these liquids to insure complete transfer of the precipitate. The crucible was ignited for 10 minutes in the flame of a Meker-Fisher burner with the use of a larger crucible underneath the Gooch crucible to prevent possible loss of the precipitate. The crucible was cooled and placed in a desiccator to await weighing.

Determination of Total Sulfate Sulfur in Urine

Reagents.

Hydrochloric acid. 1:4 solution.

Barium chloride. 5 per cent solution.

Procedure.

For the analysis of the total sulfate (inorganic plus ethereal sulfate) of the rabbit urine, the method of Folin (130) was used. A volume of urine (usually 50 ml.) representing approximately one-fifth of the total volume for the day was pipetted in duplicate into 250 ml. beakers. A solution of 1:4 hydrochloric acid was added to the urine to effect the hydrolysis of the conjugated sulfates to the inorganic form. (When the sample of urine was 50 ml. as was usually the case, the amount of hydrochloric acid was 40 ml. For smaller portions of urine, correspondingly smaller quantities of acid were added in order that the acidity might be approximately the same in all determinations.) The samples were placed on the steam bath for an hour, removed, diluted with 50 ml. water and allowed to stand overnight. The sulfate in the hydrolyzed urine was precipitated by a 10 ml. portion of a 5 per cent solution of barium chloride, added drop by drop over a period of three minutes. The mixture was allowed to stand an hour, stirred and filtered through a Gooch crucible which had been previously brought to constant weight. The precipitate was washed with 100 ml. of water and a few ml. of alcohol, using these liquids to rinse the beaker and thus insuring complete transfer of the precipitate from the beaker to the crucible. The crucible was ignited for 10 minutes in the flame of a Meker-Fisher burner, cooled, and placed in a desiccator for future weighing.

The organic sulfur content of the urine was ascertained

by subtracting the sulfur present as total sulfate from the total sulfur content of the 24-hour specimen.

Determination of Keto Acids in Urine.

Reagents.

2,4-Dinitrophenylhydrazine reagent. A 0.2 per cent solution in 2N-hydrochloric acid. Prepared by the solution with warming of 0.2 gm. of the hydrazine in 70-80 ml. of the 2N-acid and dilution to 100 ml.

Borate buffer. 8.0 gm. borax and 3.0 ml. concentrated hydrochloric acid per liter of solution.

Benzene. C. P. Thiophene-free.

Ethyl acetate. Anhydrous.

Sodium hydroxide. 2N.

Procedure.

The method for the estimation of keto acids was essentially that of Case (131) as modified by Shacter (132) and Knauff (133). This involved the reaction of the keto acids in the urine with the 2,4-dinitrophenylhydrazine reagent and the subsequent development of a red color by the addition of strong alkali to the hydrazones which had been formed. The sample of urine to be analyzed for keto acid content represented about one per cent of the total daily output except in cases of increased excretion when a smaller quantity was used. The diluted urine was placed in a 125 ml. separatory funnel and made up to 10 ml., when necessary, with water. A sample of 10 ml. of water served as a blank which was run with each determination. One ml. of the 2,4-dinitrophenylhydrazine reagent was added and the funnel shaken to insure

mixing. The solution was allowed to stand for 20 minutes to permit complete reaction. A total amount of 25 ml. of benzene was added to the funnel and the extraction carried out for three minutes. The aqueous layer was then drawn off and placed in another separatory funnel for later study. A portion of 2 ml. of water was added to the first funnel and drawn off, without shaking, to wash the stem of the funnel. This was added to the original aqueous layer now in the second funnel. The benzene layer was washed with 5 ml. of water by shaking for 30 seconds and again the aqueous layer was drawn off and collected in the second funnel. A second washing of the funnel stem with 2 ml. of water was added to the previous washings.

The benzene layer was then extracted with 10, 5, and 5 ml. portions of the borate buffer with shaking for one minute with each addition of the buffer. Between extractions small amounts (2 ml. each) of the buffer were added to the funnel and drawn off without shaking to wash the funnel stem. All of the borate extracts and washings were collected in a 25 ml. volumetric flask and the total volume was made up to 25 ml. with borate solution. The contents were well mixed before duplicate samples of 10 ml. each were transferred to 25 ml. volumetric flasks. To each 10 ml. aliquot was added 10 ml. of 2N-sodium hydroxide and the solution was made up to 25 ml. with water. The solution was thoroughly mixed and allowed to stand 90 seconds before the red coloration which developed with the addition of the alkali was measured in the Evelyn

photoelectric colorimeter with a 420 filter.

The analysis of the original aqueous layer and washings of the benzene layer is the modification suggested by Knauff (133). He found that, although the hydrazone of pyruvic acid would be completely extracted by the benzene, the hydrazone of α-ketoglutaric acid was not extracted from the aqueous solution by this organic solvent. Ethyl acetate, on the other hand, will extract this hydrazone completely. The aqueous residues from the benzene extraction were, therefore, saved and subsequently extracted with 25 ml. ethyl acetate. Borate buffer extractions of the ethyl acetate layer and subsequent analysis of the borate extract were carried out in exactly the same manner as has been described for the benzene layer. In the tabulation of the data the values for keto acid content as determined by extraction with each of the organic solvents have been included. Total keto acid concentration may be determined by summing these two values.

Standard curve for the keto acid determination.

For a standard curve a known amount of a purified keto acid was taken through the procedure as outlined above. A sample of sodium phenylpyruvate which had previously been synthesized in this laboratory by Papageorge was recrystallized and dried. A standard solution was then prepared and an appropriate range of dilutions of this standard was used in the determination of the absorption coefficient. Duplicate portions of the old sample of sodium phenylpyruvate were recrystallized and used in the preparation of standard solutions.

The K values for the two samples were found to be 2.45 and 2.70, and an average of these two values, 2.58, was taken as the K value to be used in the determinations of keto acid excretion. This is believed to be sufficiently accurate for the purposes of this work since the comparative and not the absolute values of normal and experimental keto acid excretion are of interest. It might be mentioned that another approximation is introduced by the use of phenylpyruvic acid in the establishing of the standard curve. It is possible that various keto acids may not have the same K value as this particular keto acid. The use of the one acid as a standard is justified according to the work of Waelsch and Miller (138) and of Shen and Lewis (124) who showed that of the several keto acids tested, the K values varied only ± 10 per cent.

The values for the standard curve are given in Table IX.

Determination of Creatinine in Urine.

Reagents.

Picric acid. Saturated aqueous solution.

Sodium hydroxide. 10 per cent solution.

Procedure.

Creatinine excretion was measured by means of the Jaffe reaction. In the determination creatinine is allowed to react with picric acid to form creatinine picrate. Then, on the addition of alkali, a red color develops due to the formation of the sodium salt of creatinine picrate. The

TABLE IX

STANDARD CURVE FOR KETO ACID DETERMINATION

Sample no.	Phenylpyruvic acid in 10 ml. sample	G'=G	L	K
I	mg. 0.025	86^3	0.0617	2.47
		87^0	0.0605	2.42
	0.050	75^0	0.1249	2.50
		75^1	0.1235	2.49
	0.100	57^0	0.2441	2.44
		57^1	0.2422	2.42
	0.200	33^0	0.482	2.41
		32^3	0.485	2.42
			Average	2.45
II	0.025	85^2	0.0680	2.72
		85^2	0.0680	2.72
	0.050	73^3	0.1322	2.64
		74^0	0.1308	2.62
	0.100	53^2	0.2716	2.72
		53^1	0.2736	2.74
	0.200	28^2	0.545	2.72
		28^2	0.545	2.72
			Average	2.70

Average K value = 2.58

Stock solution = 50.0 mg. phenylpyruvic acid/liter
i.e. 62.2 mg. sodium phenylpyruvate-H_2O per liter

intensity of the red coloration may be measured in the colorimeter. In this work the determination of creatinine in rabbit urine was carried out on a 5 ml. sample of diluted urine representing about 1/250 of the 24-hour volume. The blank which was run with each determination was 5 ml. of water.

The sample was placed in a 50 ml. volumetric flask and portions of 10 ml. of a saturated aqueous solution of picric acid and 1 ml. of a 10 per cent solution of sodium hydroxide were pipetted into the flask. The contents were thoroughly mixed and allowed to stand for 10 minutes for the color to develop. The solution was diluted up to 50 ml. with water and mixed, and the color was read in the Evelyn photoelectric colorimeter with a green 520 filter.

In order to set up a standard curve for this determination, pure crystalline creatinine hydrochloride was weighed and made up into a standard solution. Various dilutions over an appropriate range of concentrations were made and carried through the reaction. Since the color of the sodium salt of creatinine picrate does not follow Beer's Law, a K value could not be obtained. When the galvanometer readings are plotted against the concentration in mg. of creatinine in the sample, a curve is obtained. From this curve the mg. of creatinine in the sample of urine could be determined directly and, with the use of the appropriate factor, the excretion for the 24-hour period calculated. The rabbits used in this work were found to excrete 100-125 mg. creatinine daily.

Determination of Total Lipides in Liver.

Reagents.

Ethyl alcohol. Absolute.

Ethyl ether. Anhydrous.

Petroleum ether. Boiling range 40° - 60°.

Procedure.

The method for the estimation of total fat in the liver was suggested by Dr. Henry C. Eckstein. The liver was excised, minced, weighed and placed in a flask containing 250 ml. of absolute alcohol. It was allowed to stand for three to four days and the alcohol was then decanted into another flask. The liver was extracted with three portions of boiling absolute alcohol of 150 ml. each, these extracts were added to the original alcohol extract and the entire volume evaporated almost completely. The liver was then extracted with three portions of boiling ethyl ether of 150 ml. each and these extracts were added to the alcohol residue. The ether was evaporated as completely as possible. The fat residue was then taken up by alternate extractions with petroleum ether and aqueous alcohol and the two solvents were placed in a separatory funnel. The alcohol layer was drawn off and allowed to fall drop by drop through a layer of petroleum ether in a separatory funnel below. The alcohol layer in the lower funnel was then discarded and the two petroleum ether layers combined in a weighed Soxhlet flask. The petroleum ether was driven off by evaporation and the flask allowed to stand in a vacuum oven at 40° C. for 24 hours. The flask

and fat residue were then weighed and the weight of the fat determined by difference.

TABLE X

LIPIDE CONTENT IN LIVER OF RABBITS FED ETHIONINE

Weight of rabbit	Ethionine administered	Liver	
		Weight	Lipide content
kilo.	gm.	gm.	per cent
2*	1.45	36.0	15.6
3.2	1.10	60.9	12.0
1.6	0.73	30.4	9.4
2.3	0.73	74.4	7.4
2.6	0.73	61.3	14.1

*approximate weight

Normal lipide content of rabbit liver = 2.3 per cent (136).

TABLE XI

STANDARD CURVE FOR THE DETERMINATION OF METHIONINE AND ETHIONINE BY THE McCARTHY-SULLIVAN REACTION

DATA FOR FIGURE 1

Amino acid	Weight amino acid in sample	Average galvanometer reading
	mg.	
Methionine	0.20	81[1]
	0.40	64[2]
	0.60	52°
	0.80	41[1]
	1.00	31°
Ethionine	0.22	78[2]
	0.44	62°
	0.65	48°
	0.87	39[2]
	1.09	29[1]

The stock solutions contained equimolar amounts of the two amino acids:

0.200 gm. methionine per liter
0.218 gm. ethionine per liter

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