CYSTINURIA

by

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and

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NORMAL METABOLISM OF SULPHUR-CONTAINING AMINO ACIDS.

Although a complete picture of the normal metabolism of sulphur-containing amino acids has not yet been obtained, recent experiments, particularly those using radio-active isotopes as tracer elements, have given us a much clearer understanding of the reactions involved. Methionine and cystine (or cysteine) are the most important and probably the only sulphur-containing amino acids occurring naturally in tissues, and of these a dietary source of the former is essential. About one sixth of the minimum requirements of methionine on a cystine-free diet can be replaced by cystine (Womack and Rose, 1941) so that cystine has, to a limited extent, a methionine sparing action. There is, however, no evidence that cystine is converted into methionine in the body although the latter, as was first shown by Brand, Cahill and Harris (1935), is a source of cystine.

The first step in the metabolism of methionine may be either its reversible deamination to the corresponding keto acid, or its demethylation to homocysteine. Homocysteine can be metabolised by tissues with the formation of cysteine and du Vigneaud (1942) has reviewed most of the evidence in favour of its production in tissues. Not only can it form cysteine, but it can also replace methionine in the diet provided that an adequate supply of suitable methyl donators such as choline is also present (du Vigneaud, Chandler et al., 1939). Nevertheless homocysteine (or homocystine) has not yet been isolated from natural sources. On the other hand both liver and kidney slices readily deaminise

methionine and although Floyd and Medes (1943) did not obtain any cysteine from the keto acid of methionine, the amounts obtained when methionine or homocysteine were used as substrates were of doubtful significance. Borek and Waelsch (1942) have suggested that deamination is the first step in metabolism and that the formation of the keto acid of methionine labilises the molecule so that there is a simultaneous yielding of both methyl group and sulphur to the appropriate acceptors - that is that homocysteine itself is not an actual intermediate.

It has been shown that radio-active sulphur incorporated in methionine can be recovered in cystine and demethylation appears to be an obligatory step in this process (du Vigneaud et al., 1944). But C^{13} which was incorporated in the carbon chain of the methionine, was not detected in the cystine. On the other hand when serine labelled with N^{15} was fed to rats, a large proportion of the isotope - five times as much as was present in any other amino acid except serine - was found in the cystine (Stetten, 1942). There therefore seems no doubt that in the synthesis of cystine in the body serine supplies the carbon chain while the sulphur is derived from methionine. Brand et al., (1936) were the first to suggest that cystathionine might be formed as an intermediate in this reaction. Binkley and colleagues (1942) isolated an enzyme system from rat liver slices which converted cystathionine to cysteine, and with du Vigneaud (Binkley and du Vigneaud, 1942) it was shown that the same enzyme extract would not attack methionine but could form cysteine from homocysteine

if serine were also present. Later, (Binkley, 1944) it was clearly shown that cystathionine in the presence of adenyl pyrophosphate could be broken down to yield cysteine and phosphohomoserine. Du Vigneaud et al. (1942) had already shown that cystathionine could replace cystine but not methionine in the diet; in other words it could not form homocysteine, which, in the presence of suitable methyl donors, can replace methionine (du Vigneaud et al., 1939).

There is almost certainly an equilibrium between cysteine and cystine in the body which is brought about through the cytochrome system (Medes, 1939). Nevertheless there is also some circumstantial evidence that the metabolism of cystine differs from that of cysteine. Some of the sulphur from the sulphur-containing amino acids is converted to taurine which with cholic acid occurs in the bile acid taurocholic acid, but whether cystine or cysteine is the immediate precursor has yet to be determined. This preliminary oxidation of the sulphur inhibits the final oxidation which normally produces inorganic sulphate. For example the rate of oxidation to inorganic sulphate of some derivatives of cystine decreased in the following order: cysteine; cystine; cystine disulphoxide; cysteinesulphinic acid; cysteic acid; indicating that this series does not represent the path of oxidation leading to sulphate formation. On the other hand recovery of total sulphur also decreased suggesting that the more highly oxidised members might be more readily available for some other physiological function, e.g. taurine formation (Medes,

1937; Virtue et al., 1939).

Several possible mechanisms have been suggested for the production of sulphate from cysteine. Fromageot et al. (1940) working with liver from dogs, came to the conclusion that the following reaction occurred:-

3 cysteine $\rightleftharpoons$ cystine + H_2S + alanine

Smythe (1942) could not confirm the equation, and his results were more in accord with:-

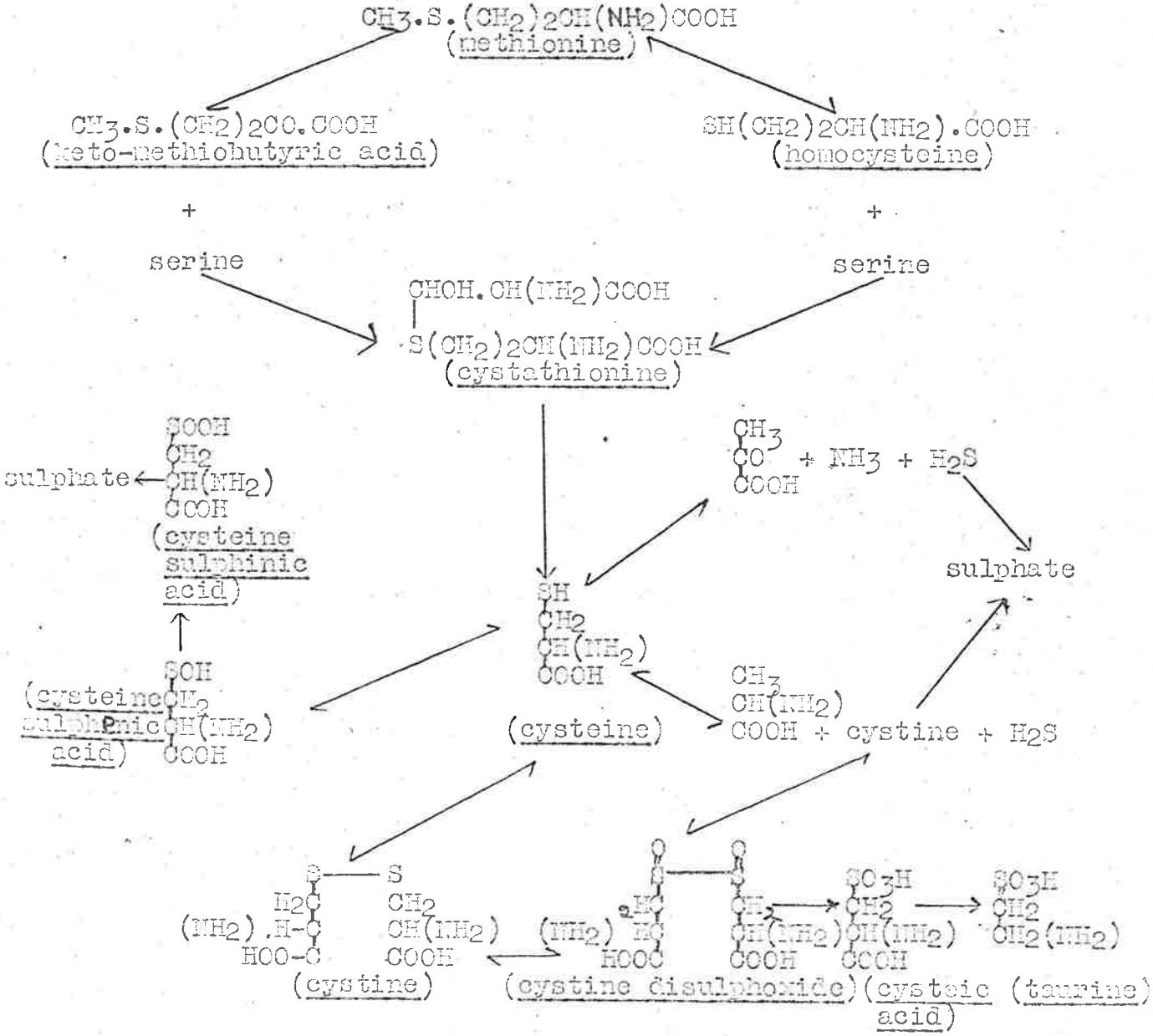
cysteine $\rightleftharpoons$ pyruvic acid + H_2S + NH_3

though in a later paper (Smythe, 1945) he suggests that some alanine may also be formed. By using H_2S containing radio-active sulphur Smythe and Halliday (1942) showed that the reaction was to some extent reversible - that is that some of the tagged sulphur was found in the cysteine. Smythe (1943) has also shown that rat liver and kidney can convert sodium sulphide to sulphate.

The existence of two cysteine oxidases and a sulphinic oxidase has been reported (Hedes, 1939, and Bernheim and Bernheim, 1939) and a scheme for the breakdown of cysteine by these and similar enzymes has been drawn up by Hedes and Floyd (1942) and has been incorporated in the diagramatic representation of the metabolism of the sulphur-containing amino acids. In this respect it is of interest to note that Bennett (1937) has found that both cystine disulphoxide and a derivative which would be expected to give rise to cysteine sulphenic acid in the body had some methionine sparing action on rats on a low methionine diet. Cysteine sulphinic acid was inactive.

Whatever the exact pathway of cystine and methionine metabolism almost all the sulphur is eventually oxidised and appears in the urine as inorganic sulphate.

The following tentative scheme for the metabolism of the sulphur-containing amino acids can therefore be drawn up.



CYSTINURIA.

In normal persons about 90% of the urinary sulphur occurs as inorganic and ethereal sulphates. These are derived chiefly from the decomposition of proteins and usually amount to about 1 gm. sulphur a day, but vary with the total protein consumed. A small and more or less constant amount - roughly 0.1 gm. sulphur per day - of neutral sulphur is also excreted, most of which is in the form of cystine, methionine and taurine (in bile salts) which each occur in small amounts in normal urine. The ingestion of large amounts of free methionine will augment the urinary methionine a little but ingested cystine is recovered only as extra sulphate. Urinary cystine is increased however, by the injection of cystine or cysteine (Blum, 1904).

In cystinuria cystine excretion is greatly increased, mainly at the expense of the urinary sulphate so that the cystine sulphur alone may exceed 200 mg. a day and the neutral sulphur may account for 40% of the total urinary sulphur. In many cases this excessive excretion is accompanied by the deposition in the urine of the typical hexagonal crystals of cystine. Except, perhaps, in children, the condition itself appears to be benign, but owing to the low solubility of cystine there is a marked tendency for cystinurics to develop calculi in the kidney or bladder. Therapeutic measures have therefore been confined to an attempt to prevent stone formation by administering alkalis to increase the solubility of cystine, by reducing the protein consumed and hence the intake of possible precursors of urinary cystine and by

taking all possible steps to prevent urinary infection. The metabolic error is not always restricted to cystine and cases have been reported in which there was a concurrent excessive excretion of tyrosine and leucine or the diamines putrescine and cadaverine. Cystinuria has also been detected and studied in dogs (Green et al., 1936; Hess and Sullivan, 1942(b) and 1943).

REVIEW OF LITERATURE.

Garrod in his book "Inborn Errors of Metabolism" has reviewed most of the early literature on cystinuria. Cystine itself was first isolated in 1810 by Wollaston from a urinary calculus (hence the name cystine). It was not, however, till 1888 that Goldmann and Baumann recognised that cystine was an amino acid containing sulphur, nor till 1899 that it was isolated from proteins, in particular from hair, and became readily available for experimental purposes. Garrod, in his resumé of the results obtained by different workers up to 1923 states that on a diet containing moderate amounts of protein 0.3 to 0.5 gm. cystine was excreted each day by most cystinurics. Many of the methods then available for the estimation of cystine were not very satisfactory and the precipitation methods in particular are known to give low results, so these figures are probably too small. Nevertheless the results were of the same order as those obtained in more recent experiments, as also was the ratio of urinary cystine sulphur in mg. to total nitrogen in gm. which normally is about 0.5 but in most cystinurics lies between 5 and 20.

Some of the early workers found, also, that there was considerable variation in the degree of cystinuria in the same individual at different times. This was sometimes correlated with variations in protein intake, and sometimes with the administration of alkalis. In other cystinurics, however, the cystine excretion remained more or less constant for a wide range of conditions. With one exception in the cases reviewed by Garrod (that of Loewy and Neuberg, 1904) - and in this particular the exception differs from all other known cystinurics - the ingestion of free cystine was not associated with a rise in urinary cystine, although the latter was increased when the protein intake was raised. In order to explain this it was suggested that cystine in protein was absorbed as a complex which the cystinuric was unable to break down in the normal way, although cystine itself could be oxidised to sulphate. In support of this theory was the observation that in some cystinurics free cystine could not always be detected in the freshly voided urine but appeared if the urine were allowed to stand for several hours and usually reached a maximum within 48 hours, though occasionally a positive test for cystine was only obtained after several months. This certainly suggested that cystine may occur as a complex in some cystinuric urines, but the original supposition, that cystine from protein is absorbed as a complex, is no longer necessary, as it is now well known that dietary methionine and not cystine is one of the chief sources of urinary cystine.

With the discovery of methionine in 1923, and with the development of better methods of determining cystine, the metabolic defect in cystinurics became more easily understood, and a study of it has contributed materially to our present knowledge of normal sulphur metabolism. From a chemical point of view it was difficult to see how methionine could be converted into cystine and the first demonstration that such a conversion did occur was made by Brand, Cahill and Harris (1935) who showed that in a cystinuric individual methionine, in contradistinction to cystine, markedly increased the urinary cystine. This hypothesis was later confirmed by the use of isotopic sulphur.

Quite a number of investigators have studied the effect of the various S-containing amino acids on cystinuria and their results have been summarised in the accompanying table (Table 1). From these it is clear that the error in cystinuria lies in the abnormal metabolism of cysteine (whether free or combined in protein as in reduced lactalbumin) and methionine. In no case did the extra cystine obtained after feeding methionine exceed that obtained from a comparable dose of cysteine which suggests that the activity of methionine is related to its ability to form cysteine. Homocysteine which has been cited as a probable intermediate in this conversion (though it has not been detected during the reaction) has, under the same conditions as methionine, also been shown to give rise to extra urinary cystine. The relative effects of homocysteine and cysteine have not been determined in the same patient at the same time, but the work

of Brand et al. (1935) indicates that homocysteine is no more effective than cysteine in producing extra urinary cystine. Hydroxy-methiobutyric acid (but not γ-thiobutyric acid) also yielded extra cystine and sulphate, though less than did corresponding amounts of methionine or homocysteine (Brand, Block and Cahill, 1937).

The effect of methionine in increasing the degree of cystinuria has been found to decrease with increasing protein intake (Lewis et al., 1936; Lough et al., 1941; Brand, Cahill and Kassell, 1938) while sulphate excretion was increased, indicating that utilisation of methionine was more efficient at higher levels of protein intake. Lewis et al. (1936) also obtained less cystine from cysteine on a high protein diet, but sulphate excretion was also lower and the result could therefore be attributed to reduced absorption.

A study of cystinurics has led to the belief that though cysteine and cystine are to some extent interconvertible, the metabolism of the two amino acids must differ. It was at first difficult to explain why cystine excreted in the urine of cystinurics should be completely oxidised when fed to the patient, and, as already explained, it was suggested that cystine in protein was absorbed as a complex and that the metabolic error was due to a failure to oxidise this complex normally. Brand, Block, Kassell and Cahill (1937), however, showed that both methionine and cystine in proteins were metabolised in the cystinuric individual in the same way as the free amino acids, and that if the cystine in a

protein were reduced to cysteine then there was a marked increase in cystinuria (Kassell et al., 1938). There therefore seems no doubt that failure to dispose of the cysteine leads to its accumulation and that the extra cysteine is converted into cystine probably during its passage through the kidney.

In most cystinurics sulphur-free amino acids have failed to increase cystine excretion. Serine gave a negative result in a cystinuric reported by Brand and Cahill (1935); glutamic acid did not increase cystinuria in the case studied by Robson (1929) and glycine given with glutamic acid did not affect the cystinuric studied by Andrews et al. (1935) who, incidentally, gave a rather weak response to methionine. One of two cases reported by Hess and Sullivan (1942a) showed increased cystine excretion when glutamic acid, glycine and, more particularly, alanine were fed, though none of these were as effective as methionine. The figures given in their paper do not, however, indicate whether the effect of the sulphur-free amino acids could be attributed to an increase in overall metabolism brought about by their calorigenic effect and not to a specific action on sulphur metabolism.

In some cystinurics a study has been made of the action of different proteins on the degree of cystinuria. The effect of increasing protein intake on the recovery of cystine from methionine, and of the reduction of cystine in lactalbumin have already been referred to. Similarly Looney et al. (1923) found that while the absolute value for cystine excretion rose with

the protein intake, the ratio of neutral sulphur to total sulphur excreted decreased suggesting that sulphur metabolism was more efficient. In addition Robson (1929) noted that extra cystine given in protein did not raise the urinary cystine level, and Andrews and Randall (1935) obtained almost the same degree of cystine excretion when the low protein intake (33 gm. per day) was increased by including 55 gm. egg protein in the diet - an increase which almost doubled the nitrogen excretion. Again Hickmans and Smallwood (1935) found that on a low protein diet cystine excretion was not correspondingly reduced, though it could be almost doubled by feeding large quantities of meat. They therefore assumed that urinary cystine was endogenous in origin and that it could be augmented by feeding extra protein because the latter stimulated endogenous catabolism.

Looney (1923) using a colorimetric method of assay, found that alkalis depressed the excretion of cystine. This, however, has not been confirmed with other cystinurics (Lewis and Lough, 1929; Robson, 1929; Brand, 1934) and is not apparently of general occurrence. Another factor which may influence cystine excretion is exercise which Hickmans and Smallwood (1935) found was associated with an increase in cystine excretion which was not accompanied by a corresponding rise in nitrogen. This effect has not been studied by other investigators and may account for some of the discrepancies reported in the literature, particularly as most cystinurics were not confined to bed during the collection of urine specimens.

EXPERIMENTS AND RESULTS

The experiments reported in this thesis have been carried out on two cystinurics and include a study of cystine, methionine and sulphate excretion after the ingestion of various amino acids and proteins. Two papers on this work have been published (Cleland 1945 and 1946) and a third has been accepted for publication. A full discussion of the results will be found in the accompanying reprints (Case I: Cleland, 1945 and unpublished paper; Case II: Cleland, 1946). For convenience the results of the effect of proteins on cystinuria have been summarised in Table II.

Cystine: In neither of the two cystinurics examined did ingested cystine increase the excretion of this amino acid, though there was a marked rise in urinary sulphate. A low recovery of extra sulphate was associated with a low nitrogen excretion and this supports the suggestion that a greater proportion of dietary cystine is used for tissue replacement when the protein intake is low, particularly after a debilitating illness as had occurred in both these cases. When the experiment was repeated on Case I eighteen months later the nitrogen excretion was nearly twice as high, and more than the theoretical amount of extra sulphur was excreted as sulphate after the ingestion of a small amount of cystine. This excessively high result for extra sulphate is believed to be due to the concurrent increase in protein catabolism indicated by a rise in urinary nitrogen on the day on which the amino acid was fed. In all cases there was a marked rise

in the ratio of sulphate sulphur to nitrogen excreted in the urine, while the ratio of cystine and methionine sulphur to urinary nitrogen remained unchanged or tended to fall.

These results are in agreement with those found for other cystinurics, namely that cystine is metabolised normally.

Cysteine Hydrochloride: In contrast to cystine the ingestion of cysteine hydrochloride was associated with a small rise in cystine excretion in Case II and a marked increase in Case I. In the former individual 5% of the ingested amino acid could be recovered as extra cystine, but as this was accompanied by a corresponding increase in urinary nitrogen it could be attributed to an increase in protein catabolism as a whole and not to a specific effect on cysteine. In this respect this cystinuric differs with one exception (Hess and Sullivan, 1942a) from other cystinurics. Nearly half the ingested cysteine could be recovered as extra sulphate - more than was obtained after the ingestion of equivalent amounts of cystine. This difference is probably at least partly due to the more rapid absorption of cysteine hydrochloride.

In Case I on a low protein diet 56% of the ingested cysteine hydrochloride was excreted as sulphate but there was only a small increase in urinary cystine (when results were expressed as cystine excreted per gram of nitrogen, the increase was more conspicuous). When the protein intake was raised to moderate levels there was a small increase in the recovery of cysteine as sulphate (66%) and another 20% could be obtained as

cystine. The initial low recovery is, I believe, due to the utilisation for tissue replacement and repair of much of that fraction of cysteine which is usually excreted as cystine since at this time protein consumption was low and requirements were probably high. I therefore consider that, since the ratio of cystine to nitrogen excreted after cysteine was greater at the lower level of nitrogen excretion, these results are not in disagreement with the suggestion that sulphate production from cysteine is more efficient when the protein intake is raised.

Methionine: In both cystinurics after the ingestion of methionine the amount of this amino acid recovered unchanged in the urine was within the normal range. In Case I, however, more than 20% could also be recovered as extra cystine while in Case II the increase in cystine was so small that it is doubtful whether it has any significance, particularly as there was no alteration in the ratio of cystine excreted per gram of nitrogen. Recovery as sulphate in the latter case amounted to 66%, indicating fairly efficient oxidation of the methionine. Since the protein intake was low, the 20% of ingested methionine not accounted for was probably utilised for tissue replacement.

In Case I not only was the cystine excretion unusually high, but the sulphate excretion was low and, in all, only about 60% methionine could be accounted for; presumably the rest was required by the tissues. (In this patient recovery of sulphate from all three sulphur-containing amino acids was low when the

average nitrogen excretion was low). On the other hand when the experiments were repeated eighteen months later and the patient was eating a moderate protein diet the increased excretion of the three sulphur fractions examined was high after the ingestion of methionine, and, in the case of sulphate excretion, exceeded the theoretical yield. The excess was probably due to the concurrent rise in nitrogen excretion, and for this reason the recovery of all three sulphur fractions is probably too high. Even taking this into consideration the results show a more efficient utilisation of methionine when the protein intake was increased. The figures, however, are not strictly comparable with those obtained in 1945 since a smaller test dose of methionine was given in the more recent experiments.

Homocystine: Case I, after the ingestion of 1.1 gm. homocystine, showed a rise in cystine, methionine and sulphate excretion although there was a fall in urinary nitrogen. Two other cystinurics failed to excrete extra cystine after homocystine (see Table I) although there was a rise in urinary sulphate. One of these, and another cystinuric excreted extra cystine after taking homocysteine. In the present case total recovery of sulphur was not very high, most of it appearing as extra cystine or methionine and a little less as sulphate. These low results are in part attributable to the concurrent fall in nitrogen excretion since sulphate excretion per gram of nitrogen was comparable with that obtained after equivalent amounts of methionine or cystine while the cystine : nitrogen and methionine : nitrogen

ratios were higher. The effect on methionine excretion is particularly interesting and suggests either competitive inhibition of methionine breakdown by homocystine or the conversion of homocystine to methionine. From evidence already produced it has been suggested that homocysteine is not a normal metabolite and in this respect it is of interest to note that although similar amounts of cystine were excreted, extra sulphate recovered from homocystine was less than from equivalent amounts of methionine - a result which would not be expected if homocysteine were a true intermediate.

Glycine: Glycine, as an acceptor of methyl groups, might be a limiting factor in methionine metabolism. If so its ingestion should be accompanied by an increase in urinary cystine. Though in both cases studied there was a rise in cystine excretion, this was associated with an equivalent increase in urinary nitrogen and methionine while the increase in sulphate was relatively greater. Since the rise in sulphate excretion was not at the expense of the cystine and methionine, it is unlikely that glycine specifically influences methionine or cysteine metabolism. The rise in sulphate may, however, be attributed to an increase in the catabolism of proteins rich in cystine.

Again when glycine was given in conjunction with methionine in Case I the increase in sulphate excretion was high (78% ingested methionine) and with the extra cystine (16.8%) and methionine (10%) the recovered sulphur exceeded the theoretical amount. The excess is probably due to the stimulation of sulphate

excretion by the glycine. Although recovery of extra methionine was rather low there was a high ratio of methionine to urinary nitrogen, so that the presence of glycine does not appear to increase the rate of methionine breakdown.

Alanine: The ingestion of alanine, on the other hand, increased the relative excretion of cystine, but did not alter either methionine or sulphate excretion. This effect is difficult to explain, particularly as it was not associated with an alteration in other sulphur fractions. Though alanine may be formed during the production of H_2S from cysteine (Fromageot et al., 1940; Smythe, 1945) it does not inhibit these reactions in vitro.

In contrast, when alanine was given with methionine in Case I the excretion of extra cystine and methionine and the increase in cystine and methionine excreted per gram of nitrogen were lower than when the alanine was replaced by leucine, glycine or serine, and the excretion of extra sulphate was high (78% methionine). This effect may possibly be related to the increased efficiency of methionine oxidation at high protein levels. Perhaps in the presence of excess alanine (or a high protein intake) the methionine undergoes some different metabolic change or the alanine inhibits one of the steps which normally lead to the production of cysteine.

Serine: Serine could affect the metabolism of the sulphur-containing amino acids either by acting as sulphur acceptor from methionine or by inhibiting liver desulphurase which converts cysteine to H_2S. In the former case one would expect, when serine was fed, to find an increase in cystine and a reduction in methionine but no

alteration in sulphate excretion; in the latter case methionine excretion would be unchanged, cystine higher and sulphate lower. Results obtained with Case I indicate that serine may inhibit cysteine oxidation in the cystinuric.

When serine was given in conjunction with methionine the recovery of the latter as cystine, methionine and sulphate amounted to 16, 14 and 59% respectively, values which, owing to a concurrent rise in nitrogen, are probably too high. Sulphate excretion per gram of nitrogen was also low, but cystine excretion was not correspondingly increased (as was found when serine alone was given). The inhibition of sulphate production and cysteine destruction was therefore not as great when serine was given with methionine, possibly because some of the serine was converted to homoserine during the metabolism of methionine.

Leucine: Leucine was used as a control amino acid in these experiments because it is biologically rather inert and is not known to be involved in the metabolism of the sulphur-containing amino acids. It was therefore surprising to find that in Case I although sulphate and methionine excretion were not affected, the ingestion of leucine was associated with an increase in cystinuria.

Similarly when leucine was given in conjunction with methionine the per cent recovery of the latter as cystine was greater than when leucine was replaced by other amino acids, and the recovery as sulphate was rather low (59%) although the excretion of sulphate per gram of nitrogen was high - in this

respect differing from the action of serine in which low sulphate excretion was associated with a low sulphate : nitrogen ratio. Leucine, therefore, appears to restrict the oxidation of cysteine to a small degree.

Proteins: In order to reduce the concentration of cystine in the urine of cystinurics it is usual to advise the consumption of a diet low in protein. In some cystinurics, however, a low protein intake has not been accompanied by a corresponding reduction in cystine excretion. The effect of different proteins on cystine and methionine excretion in the two cystinurics studied has been summarised in Table II.

In Case I on a low protein diet the methionine and cystine excretion remained almost constant when the two eggs in the basal diet were replaced by equivalent amounts of gelatine (which contains little cystine or methionine) or meat (which contains about ¾ methionine and ½ cystine present in eggs). Even when the protein intake was increased to moderate levels there was an increase in sulphate but no alteration in methionine and cystine excretion and replacement of the two eggs by gelatine at this level resulted in an increase, and not the expected decrease, in urinary cystine. The excretion of cystine and methionine per gram of nitrogen were both lower at the higher level of protein consumption, indicating more efficient utilisation of these amino acids when the protein intake was raised to moderate levels. In this cystinuric, then, the inclusion of proteins rich in cystine and methionine in a diet containing little or moderate amounts of

protein did not increase the degree of cystinuria.

In Case II similar results were obtained with eggs and gelatine - that is, the replacement of 2 eggs by gelatine on a moderate protein intake increased the degree of cystinuria. Addition of gelatine to the basal diet containing the eggs did not cause as great an increase in cystinuria as did substitution. Methionine excretion was not greatly affected, but tended to be high when gelatine replaced the eggs.

On the other hand replacement of eggs by casein (which contains about the same amount of methionine as eggs but very little cystine) resulted in the greatest excretion of both cystine and methionine and the greatest ratio of cystine and methionine excreted per gram of nitrogen. Considerably less cystine and methionine (and a lower ratio of excretion per gram of nitrogen) were obtained when casein was given in addition to the eggs, though, in the case of cystine, the excretion was higher than that obtained when an extra 2 eggs (equivalent to the added casein) were given.

From these results it can be seen that although cysteine and methionine may be the source of urinary cystine in cystinuria, the amount excreted is by no means proportional to the intake of these amino acids, and that the excretion of sulphur-containing amino acids may be greater at low than at moderate levels of protein intake while the efficiency of utilisation is correspondingly reduced. In particular it should be noted that the cystinuria was least when, at moderate levels of protein intake, the major source of protein was eggs which are an excellent source of both

cystine and methionine; and that, in the one case studied, replacement of eggs by casein which contained almost the same amount of methionine but much less cystine was accompanied by an increase in cystinuria. This suggests that the presence of dietary cystine inhibits the excretion of cystine (in both cystinurics the ratio of cystine excreted per gram of nitrogen tended to fall when cystine was ingested). It must, however, be remembered that eggs contain factors other than protein which may influence methionine metabolism.

The excretions of sulphur-containing amino acids when the protein intake was high were not determined.

DISCUSSION.

An analysis of these results and of those obtained from other cystinurics indicates that cystinuria is the result of some defect in cysteine (and not in cystine) metabolism; and that methionine, in that it is a potential source of cysteine, may also be responsible for cystinuria. The degree of cystinuria, however, may be influenced by factors other than the sulphur-containing amino acids, for example the intake of other amino acids (leucine, serine and alanine, but not glycine) or some substance present in eggs. Little, however, has been said about the point in metabolism at which the defect occurs - a defect which is by no means complete since at least some of the ingested cysteine is oxidised and appears in the urine as inorganic sulphate.

The excessive excretion of cystine could be due to an

abnormally low renal threshold or to an increased production or to limited destruction of cysteine or to all three.

If cystinuria were due solely to increased production of cysteine then it would be expected that sulphate excretion would remain normal, and, if methionine were the source of extra cysteine, then methionine excretion would be reduced. In all cystinurics studied the sulphate excretion was unusually low, so that excessive cysteine production cannot be the only cause of cystinuria, though it could be a contributing factor. However, if the rate of methionine destruction were unusually high and therefore excessive cysteine production was a contributing cause, then one would expect that in the cystinuric individual methionine excretion would be correspondingly low and that little if any unchanged methionine would be recovered after the ingestion of a test dose of this amino acid. Andrews et al. (1935) found that methionine excretion in a normal and a cystinuric boy were similar and in the experiments submitted for this thesis methionine excretion and the per cent of a test dose of methionine recovered unchanged in the two cystinurics studied was of the same order as that obtained for normal individuals. The action of homocystine in increasing methionine as well as cystine and sulphate excretion indicates that it competes with methionine for the enzyme systems responsible for its metabolism, an effect which would not be expected if this mechanism were unusually rapid in its action. Similarly other amino acids which might be expected to increase the rate of destruction of methionine by supplying suitable acceptors (such

as glycine as a methyl acceptor and serine as an acceptor of sulphur) did not have any appreciable effect on methionine excretion even when taken in conjunction with a provocative dose of methionine. These results do not support the hypothesis that the conversion of methionine to cysteine is unusually rapid in the cystinuric.

One of the puzzling features of cystinuria is that cystine itself is completely oxidised. Failure to recover 100% ingested cystine as sulphate is attributed by Hickmans and Smallwood (1935) to incomplete absorption as this amino acid (unlike cysteine hydrochloride) is only sparingly soluble in water, but in any case, extra urinary cystine is not obtained. There is, however, one aspect which has not been examined. Most investigators have used either l-cystine and l-cysteine or have derived the amino acids from biological material and have not determined the rotation. Recently Albanese (1945) found that even in normal individuals 10% d-cystine was excreted in the urine unchanged. This suggests two possible causes of cystinuria, either the cystinuric is incapable of destroying that portion of d-cystine normally formed in the organism or that abnormal amounts of the unnatural acid are formed, e.g. through the keto acids of methionine and cysteine, or the breakdown of cysteine to alanine, cystine and H_2S as described by Fromageot et al. (1940). Du Vigneaud, Wood and Irish (1939) have shown that there is some inversion of benzene derivatives of d-cysteine in rats; presumably the same mechanism would convert d-cysteine to l-cysteine in normal individuals.

Assuming that surplus cystine and methionine are oxidised to sulphate by some other path this would explain why on high protein diets more of a test dose of methionine is excreted as sulphate and less as cystine. The problem appears to be somewhat analagous to phenylketonuria which can be produced in normal individuals by feeding d-phenylalanine (Penrose, 1946).

The fact that cystinuria has sometimes been associated with the excretion of tyrosine and leucine and a relatively low ratio of urinary urea nitrogen to total nitrogen, led to the suggestion that deamination of amino acids in general was involved. However the excessive excretion of sulphur-free amino acids is by no means a constant finding in cystinuria, nor has there been any other evidence to suggest that deamination was at fault. Values for amino-nitrogen given by Hickmans and Smallwood (1935) and Robson (1929) were within the normal range and did not vary appreciably with alterations in the protein intake.

Hedes (1939) has isolated from liver an enzyme system which converts cysteine to sulphate and another, possibly part of the former, which attacks cysteine sulphinic acid. As Bennett (1937) found that the sulphinic acid did not have a methionine-sparing action on a low methionine diet it is unlikely that a defect at this point would cause cystinuria. It is however conceivable that the cystinuric may be unable to bring about the initial step in this scheme of oxidation. The fate of the possible intermediate, cysteine sulphenic acid, or of the sulphinic acid in cystinuria has not been determined. As with normal individuals,

cysteic acid gives rise to no extra cystine and very little extra sulphate (Andrews et al., 1935) and cannot be considered as an intermediate.

The mechanism of H_2S production from cysteine in normal individuals is not yet clear. The suggested pathways involve the production of either alanine or the keto acid, pyruvic acid. The enzyme system responsible for the latter reaction appears to be closely related to and possibly identical with that which converts serine to pyruvic acid. Since serine was found to inhibit cysteine desulphurase (Binkley, 1943) it was thought that the ingestion of serine alone might increase cystine excretion if such a mechanism was of importance in the living animal. It is significant that in these experiments a small amount of serine was found to increase cystine and to reduce sulphate excretion. It is to be noted that other sulphur-free amino acids which increased cystine excretion did not reduce the urinary sulphate at the same time.

On the other hand if the oxidation involved the production of alanine as suggested by Fromageot et al., (1940) this amino acid might be expected to depress cysteine oxidation, though they found that cystine itself and not alanine inhibited the reaction, (Lawrence and Smythe, 1943, also found that alanine did not inhibit H_2S production from cysteine with the enzyme system they used). In both cystinurics studied by me, and in one of two examined by Hess and Sullivan, (1942a) alanine increased cystine excretion. In one of the former cases, however, when alanine was given in conjunction with methionine, more methionine sulphur was recovered

as sulphate and less as cystine and methionine than with the other sulphur-free amino acids tried - an effect simulating that of a high protein diet, and one at present inexplicable on our present knowledge of normal sulphur metabolism.

The increase in efficiency of methionine metabolism which is associated with a high protein diet suggests that some constituent of protein may be a limiting factor in the breakdown of cysteine to sulphate, though at present it has not been shown that protein intake affects similarly the efficiency of cysteine metabolism. Some complex similar to cystathionine might be formed immediately prior to the release of H_2S, and it is conceivable that at high protein levels such a mechanism becomes an important pathway for the breakdown of cysteine, and that in the cystinuric the predominance of such a reaction is responsible for the better utilisation of methionine when the protein intake is increased.

One further possibility suggests itself as a cause of cystinuria. An abnormally low renal threshold for cysteine might be responsible for an excessive excretion of cystine. Presumably the breakdown of cysteine normally occurs in the liver, or possibly in the tissues and therefore if a low renal threshold were the only cause of cystinuria one would not expect a particularly marked response to the ingestion of cysteine and no increase in cystinuria with methionine. Two cystinurics, one studied by Hess and Sullivan (1942a) and the other by me (Cleland, 1946) would fall into such a category, and the latter in particular suggests no failure in normal utilisation of the sulphur-containing amino acids while the

average daily cystine excretion was much lower than that of most other cystinurics. Further, two of three of his relations examined showed a rather high level of cystine excretion though this was still within the normal range as given by Sullivan and Hess (1936).

CONCLUSION.

From a review of the literature and from experiments submitted for this thesis the possible causes of cystinuria can be summarised. The defect appears to lie in the normal oxidation of cysteine (but not of cystine) and is not due to excessive production of cysteine from methionine. From a knowledge of normal sulphur metabolism it is suggested that the fault lies either in the abnormal formation or metabolism of the unnatural isomer of cystine or it is due to failure to convert cysteine to H_2S or cysteine sulphenic acid at the normal rate. In certain cases cystine metabolism does not seem to be seriously deranged, and it is possible that cystinuria in these cases is due to a low renal threshold.

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TABLE I.

EFFECT ON CYSTEINE EXCRETION OF S-CONTAINING AMINO ACIDS IN CYSTINURIA.

Reference	Protein Intake	Cystine	Cysteine	Homocystine	Homo-Cysteine	Methionine
Brand, Cahill and Harris (1935)	Low	-	++			++
Brand, Cahill and Block (1935)	Low			-	++	
Andrews et al. (1935)	Moderate	-				?+
Lewis, Brown and White (1936)	Moderate	-	++			++
Lewis, Brown and White (1936)	High	-	+			+
Brand, Block and Cahill (1937)	Moderate				++	++
Lough et al. (1941)	Moderate	-	++			++
Lough et al. (1941)	High			-		-
Hess and Sullivan (1942)	Moderate	-	+?			-
Hess and Sullivan (1942)	Moderate	-	++			++

TABLE II

EFFECT OF DIFFERENT PROTEINS ON EXCRETION OF S-CONTAINING AMINO ACIDS.

Chief Protein	Dietary Sulphur As		Total N (gm) Excreted	Average Excretion of		Average Excretion per gm. Nitrogen of		
	Cystine	Methionine		Cystine (gm)	Methionine (gm)	Cystine S (mg)	Methionine S (mg)	
Gelatine	Low	Low	5.2	0.45	0.33	25	12	Case I Cleland 1945.
Gelatine	Low	Low	7.8	0.56	0.33	19	8.8	
Meat	Moderate	Moderate	5.5	0.48	0.33	23	12.8	
Eggs	Moderate	Moderate	5.2	0.45	0.33	23	12.0	
Eggs	Moderate	Moderate	7.0	0.45	0.33	17	10	
Gelatine	Low	Low	7.4	0.38	0.28	13.5	8.0	Case II Cleland 1946.
Casein	Low	Moderate	6.7	0.47	0.33	18.8	10.4	
Eggs	Moderate	Moderate	6.9	0.28	0.26	11.2	8.3	
Eggs + Gelatine	Moderate	Moderate	7.9	0.31	0.26	10.5	7.0	
Eggs + Casein	Moderate	High	8.1	0.38	0.26	12.6	7.0	
Eggs + Eggs	High	High	7.2	0.34	0.28	12.5	8.3	

CYSTINE, METHIONINE AND SULPHATE EXCRETION IN CYSTINURIA

By

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Cysteine and methionine are now considered to be the chief sources of urinary cystine in cystinuria. However the excretion of extra cystine following the ingestion of given amounts of these amino-acids is variable and Hess and Sullivan (1942) have reported a case in which cystine excretion was increased when either glycine or alanine were taken, and Cleland (1946) obtained extra cystine after giving alanine (but not glycine) to a cystinuric. This paper gives the results obtained when leucine, serine, alanine and glycine as well as some of the sulphur containing amino acids were given to a cystinuric.

EXPERIMENTAL DETAILS

The cystinuric patient was the young woman on whom some investigations had already been carried out (Cleland, 1945). Since the completion of these earlier studies frequent X-ray analyses have shown no evidence of new stone formation in the kidney although the urine continues to contain cystine crystals.

Diet - During these experiments the patient, who was working, consumed her normal diet which usually consisted each day of an egg, one serve of meat, a pint of milk, cereals, fruit and vegetables. The total protein intake was about 1.2 gm. per kilo.

Amino Acid Supplements - The amino acid supplements were taken in gelatine capsules on the morning of the day indicated. In one

series of experiments the supplement consisted of 2.5 gm. methionine together with the molecular equivalent of leucine, serine, glycine or alanine taken on two consecutive days. In the other series the supplement was 1 gm. cystine or its equivalent of methionine or homocystine, 1.3 gm. serine, alanine or glycine or 2.2 gm. leucine each taken on only one occasion.

The dl-serine was obtained from Gelatin Products through the kindness of Dr. E. Ellion of Washington, and the dl-homocystine through the courtesy of Dr. V. du Vigneaud of Cornell University. Cystine was isolated from wool and the methionine, glycine and alanine came from L. Light and Co. Ltd., May and Baker and B.D.H. respectively.

Methods - The methods used were those described in previous papers (Cleland, 1945 and 1946).

RESULTS AND DISCUSSION

Results are shown in Tables 1 and 2 and Fig. I.

The excretions of extra cystine, methionine and sulphate sulphur following the ingestion of the amino acids were calculated for the days on which they were fed and the following day. Normal values were obtained from the one or two days preceding the test.

The results have also been expressed as the ratio of cystine, methionine and sulphate sulphur excreted per gram of nitrogen (Fig. I).

In comparing the effects of equivalent amounts of amino acids it should be remembered that where the nitrogen excretion is low (indicating reduced protein catabolism) values for the

recovery of extra sulphur will tend to be low and the excretion of sulphur fractions per gram of nitrogen will tend to be high while the reverse conditions hold when the nitrogen excretion is high.

The results can be discussed under three headings.

1. <u>The Effect of Single Small Doses of Sulphur-Containing Amino Acids</u>.

<u>Cystine</u> - As was found previously, the ingestion of cystine was followed by a marked rise in urinary sulphate. The extra sulphate excreted on this occasion was, however, greater than could be accounted for by the complete oxidation of the cystine, and there was also a small rise in methionine excretion. This exaggerated effect was probably due to the concurrent increase in urinary nitrogen - the average excretion being 10.2 gm. N per day after cystine and 9 gm. per day for the two preceding days. The effect of cystine can therefore be better shown when the results are expressed as cystine, methionine and sulphate sulphur excreted per gram of nitrogen (Fig. I). This shows a definite rise in the ratio of urinary sulphate to total nitrogen, an unaltered methionine: nitrogen ratio and a relative fall in cystine excretion.

These results are similar to those obtained from the same cystinuric about 18 months previously and show that ingested cystine is not a source of extra urinary cystine (cystine excretion tends to fall) but is almost completely oxidised to sulphate.

Methionine - The ingestion of an equivalent amount of methionine was also followed by a great increase in sulphate excretion but, in contrast to the effect of cystine, this was accompanied by a rise in urinary cystine and methionine. The extra sulphate excreted was greater than could be accounted for by the complete oxidation of the ingested amino acid, and this again can be attributed to concurrent variations in nitrogen excretion, the average being in this case 8.8 gm. N per day after methionine and 7.2 gm. N for the preceding day. The ratio of urinary sulphate sulphur to total nitrogen (Fig. I) after methionine was increased to about the same level as after an equivalent amount of cystine, but there was also a small rise in both cystine and methionine excretion per gram of nitrogen, the relative rise in sulphate being greater and in cystine and methionine lower than the results obtained 18 months before (Cleland, 1945). This difference is not unexpected for others (Lewis et al., 1936; Lough et al., 1941) have shown that the excretion of extra cystine following a given quantity of methionine tends to decrease with increasing protein intake, and the average N excretion during the earlier experiment was only 4.8 gm. per day.

These results confirm those obtained from the same cystinuric on a previous occasion in which methionine was found to increase cystine, methionine and sulphate excretion (Cleland, 1945).

Homocystine - If, as recent evidence suggests, the main path in the breakdown of methionine to cystine involves its demethylation to homocysteine, then it would be expected that the latter (and

homocystine) would increase cystinuria. Homocysteine has not, however, been isolated from tissues, and therefore may not be a true intermediate though its metabolic breakdown may follow the same path as that of methionine.

In the present case, after the ingestion of 1.1 gm. homocystine, there was a rise in urinary sulphate, cystine and methionine which would account for 15, 17 and 24 p.c. respectively of the ingested amino acid although the concurrent nitrogen excretion fell from 8.8 to 7.4 gm. per day. This appears to be the first record of extra cystine being excreted after the ingestion of homocystine. Lough et al. (1941), obtained no increase in cystine excretion when homocystine was given to a cystinuric patient who was consuming a high protein diet, but under these conditions methionine also was ineffective. Brand, Cahill and Block (1935), recovered about 32 p.c. ingested homocystine as sulphate and 24 p.c. unchanged but none as cystine. However when homocysteine was given to their patient both cystine and sulphate excretion rose. They also state (cf. Butz and du Vigneaud, 1932) that the Lugg-Sullivan modification for cystine determination (which was used in the present experiment) was not affected by homocystine, so that the apparent rise in cystine excretion in the present case cannot be attributed to unchanged homocystine.

Unfortunately only a little homocystine was available and although a small amount did not appear to affect the methionine titration, there was insufficient material to confirm these results. However, as, under the conditions of the titration,

neither cystine nor cysteine are oxidised it seems unlikely that homocystine would interfere with the estimation. Nevertheless it should be borne in mind that it is possible that some of the estimated methionine may really be due to homocystine.

If the method for determining methionine does not include homocystine then the increased excretion following the ingestion of homocystine could be attributed to the synthesis of methionine from homocystine; Bennett and Toennies (1946) and du Vigneaud et al. (1939) have found that under suitable circumstances rats can utilise homocystine as a source of methionine. Another explanation is that homocysteine and methionine compete for enzymes and substrates responsible for their oxidation to cysteine, so that methionine oxidation is inhibited. Similarly rate of breakdown of methionine by this means may be limited when the intake of methionine is high and cysteine production from added methionine would then be restricted. Some other mechanism would then be responsible for the conversion of methionine to the extra sulphate obtained at high protein levels. In this respect it is of interest to note that although equivalent amounts of homocystine and methionine caused similar increases in cystine excretion much more extra sulphate was obtained from methionine.

2. <u>The Effect of Single Small Doses of Sulphur Free Amino Acids.</u>

<u>Serine</u> - Binkley and du Vigneaud (1942) and Stetten (1942) have presented evidence to show that the following reaction can occur in tissues:-

homocysteine + serine ⟶ cysteine

It is therefore conceivable that an inadequate serine intake might be a limiting factor in the production of cysteine.

On the other hand Binkley (1943) and Lawrence and Smythe (1943) have shown that in some animals the enzyme which converts cysteine to pyruvic acid resembles and may be identical with that which breaks down serine and that its action is inhibited by serine. If this is so then a high serine intake might limit the destruction of cysteine and so be responsible for increased cystinuria.

When 1.3 gm. serine was given to this cystinuric there was a small rise in cystine excretion and a corresponding fall in sulphate excretion, while the urinary methionine and nitrogen were scarcely affected. The extra cystine was nearly as great as after an equivalent amount of homocystine but, as already explained, the effect of the latter has probably been underestimated and the real difference should be greater. An examination of Fig. I, where the sulphur excretion is related to urinary nitrogen, gives a better indication of the relative effects of these two amino acids.

Serine, then, did cause a small rise in cystine excretion at the expense of the urinary sulphate.

Alanine - Hess and Sullivan (1942), found that alanine increased the cystine excretion in one of two cystinurics studied. Cleland (1946), in another cystinuric, also found that extra cystine was obtained after taking alanine and suggested that it might be due to a reversal of the reaction which is responsible for the

formation of alanine in tissue slices during the production of H_2S from cysteine (though neither Fromageot (1940), nor Lawrence and Smythe (1943) found any inhibition in vitro). Another tentative explanation was that alanine might be a source of serine which might be a limiting factor in cysteine formation.

In the present case, when 1.3 gm. alanine was given, there was a fall in urinary nitrogen corresponding to a reduction in protein catabolism during that period, and this reduction may have masked any absolute increase in cystine excretion caused by the alanine. There was, however, a very definite rise in the ratio of cystine excreted per gram of nitrogen, and as this was not accompanied by a corresponding increase in the relative excretion of sulphate and methionine, it suggests that in this case, too, alanine caused an increase in cystine excretion.

Glycine - Because methionine is utilised for its methylation, glycine might be expected to influence methionine metabolism.

Luck and Amsden (1931), reported that, in dogs, parenteral administration of amino acids including glycine and alanine stimulated both protein catabolism and sulphate excretion while Reid (1939), obtained a marked increase in urinary sulphur and nitrogen but a fall in the ratio of sulphate to nitrogen excretion when dogs were given large doses of glycine and alanine, (25 and 30 gm. respectively).

Hess and Sullivan (1942), found that glycine increased cystine excretion in the same cystinuric who responded to alanine.

this was accompanied by a rise in urinary sulphate and may have been due to increased endogenous metabolism rather than to a specific action on cystine. No increase in cystinuria after the ingestion of 5 gm. glycine was obtained from the other cystinuric studied by me (Cleland, 1946) and the marked rise in urinary sulphate was accompanied by an equivalent increase in nitrogen excretion. On the other hand, in the earlier experiments on the present patient (Cleland, 1945), when gelatine (which contains large amounts of glycine) was substituted for other proteins in the diet the cystine, methionine and in particular the sulphate excretions were greater than would have been expected on theoretical grounds, and, on the medium protein diet (N excretion = 7.8 gm/day), the ratio of urinary sulphate to nitrogen was increased. In the present experiment, after a single small dose of glycine (1.3 gm.), cystine and methionine excretion were unaltered but there was a rise in the ratio of urinary sulphate to nitrogen which was not, however, as great as after smaller doses of the S-containing amino acids. In this individual, then, glycine not only increased the endogenous protein metabolism but also, and to a relatively greater extent, stimulated sulphate excretion. This apparently specific action on urinary sulphate may be merely a reflection of the type of protein being catabolised, (e.g. those rich in cystine such as serum proteins), rather than a specific action on methionine metabolism, as there was not a corresponding fall in cystine and methionine excretion.

Leucine - As leucine is biologically rather inert and has not

yet been shown to be involved in the metabolism of any of the S-containing amino acids, it was chosen as a suitable control for these experiments. It was therefore surprising to find that the ingestion of 2.2 gm. leucine was followed by a relative and an absolute rise in cystine excretion while methionine and sulphate excretion were practically unaltered. Both on this occasion and when leucine was given with methionine the nitrogen excretion was unusually low indicating that less protein was being catabolised. In addition these results only include extra sulphur excreted during the day on which the amino acid was fed. The value given for extra sulphur excreted as cystine is therefore too low. If these results are to be compared with those obtained after other amino acids and in which concurrent nitrogen excretion was appreciably higher, then it should be remembered that the value for sulphur excretion per gram of nitrogen will tend to be high when nitrogen excretion is unusually low.

Unfortunately, owing to the onset of menstruation specimens of urine were not collected on the following day and menstruation itself may have affected the results.

3. The Effect of Sulphur-Free Amino Acids in Conjunction with 2.5 gm. Methionine.

Methionine and Leucine - Although leucine was used as a control amino acid a single small dose was found to increase cystine excretion, and when it was given in conjunction with methionine the p.c. of the latter recovered as cystine (23 p.c.) or excreted per gram of N was greater than after the other amino acids tried.

The excretion of sulphate S per gram of N was also higher than after other test doses of the amino acids although this accounted for only 60 p.c. methionine; but, as already explained, the absolute S excretion is probably too low and the relative excretion too high. Concurrent methionine excretion was not affected by the addition of leucine to a test dose of methionine.

Methionine and Serine - The possibility that the variations in the p.c. recovery of methionine at different levels of protein intake might be due in part to inadequate supplies of serine has already been discussed, (Cleland, 1945 and 1946), and it was thought that the recovery of extra cystine from a test dose of methionine might be increased if additional serine were available at the same time. After taking a total of 5 gm. methionine and 3.5 gm. serine the extra sulphur recovered as cystine, methionine and sulphate amounted to 16, 14 and 59 p.c. respectively, values which, owing to a concurrent rise in nitrogen excretion, are probably too high. When compared with the results obtained after leucine and methionine, there was an almost identical recovery of extra sulphate, a trace more methionine and appreciably less cystine when serine was used. These differences are of the same order as those obtained when small amounts of serine or leucine alone were given. Though it is possible that some of the extra cystine was due to the effect of the serine this could not have been very great and so lack of serine cannot be considered as a major factor limiting the production of cystine from added

methionine.

Methionine and Alanine - As with leucine, a small dose of alanine was found to increase the urinary cystine without affecting the sulphate and methionine excretion. Yet when alanine was given with methionine the excretion of extra cystine and methionine and the increase in urinary cystine and methionine excreted per gram of nitrogen were lower than when the other amino acids were given with the methionine. The excretion of extra sulphate sulphur was rather high (78 p.c. ingested methionine) and the total extra urinary sulphur accounted for 93 p.c. of the methionine. Why alanine should restrict the excretion of cystine from methionine when by itself it increases the urinary cystine is difficult to decide. Perhaps in the presence of excess alanine the methionine undergoes some different metabolic reaction or the alanine inhibits one of the steps in its usual breakdown to cysteine. Neither of these possibilities seem particularly satisfactory. Its action on methionine metabolism was similar to that of a high protein diet, and possibly the same factors may be responsible.

Methionine and Glycine - Glycine alone was found to increase the sulphate excretion, but not urinary cystine or methionine. In conjunction with methionine the sulphate excretion was high (78 p.c. methionine) and the extra cystine (16.8 p.c.) was of the same order as after serine and methionine, and the methionine (10 p.c.) somewhat lower, though not as low as after alanine and methionine. When these results are expressed as cystine, methionine and sulphate sulphur excreted per gram of nitrogen the values

resemble those obtained after equivalent amounts of leucine and methionine. The total sulphur recovered was greater than could be accounted for by the methionine and the excess was probably due to the stimulation of sulphate excretion by the glycine.

Glycine, then, did not affect appreciably the excretion of the sulphur containing amino acids derived from added methionine, but urinary sulphate was very much greater.

SUMMARY

Total nitrogen and cystine, methionine and sulphate sulphur have been determined in the urine of a cystinuric woman.

Ingested cystine increased urinary sulphate but did not affect the excretion of S-containing amino acids.

Both methionine and homocystine when taken by mouth increased the excretion of cystine, methionine and sulphate sulphur.

Single small doses of serine, alanine and leucine did not alter methionine excretion but increased urinary cystine and in the case of serine reduced the urinary sulphate.

Glycine taken by mouth caused a marked rise in urinary sulphate but the excretion of the sulphur-containing amino acids was unaltered.

Recovery of unchanged methionine after a test dose was greatest (14 p.c.) if leucine or serine were given in addition, and least (7 p.c.) when alanine was added. After adding an equivalent amount of glycine 10 p.c. of the methionine was recovered unchanged.

The recovery of extra cystine from added methionine was

greatest (23 p.c.) when leucine was given as a supplement and least (8 p.c.) when alanine was added. When the supplement was serine or glycine 16 and 17 p.c. respectively of the methionine could be accounted for as extra cystine.

Extra sulphate after the test dose of methionine was 59 p.c. when serine or leucine were given in addition, and rose to 78 p.c. when equivalent amounts of alanine or glycine were used.

Possible interpretations of these results are discussed.

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TABLE I.

INFLUENCE OF VARIOUS AMINO ACIDS ON URINARY SULPHUR IN CYSTINURIA.

te.	Amino Acid Supplement	Volume (mils.)	Total N (gm.)	Sulphur (gm.) as Cystine	Methionine	Sulphate
ch 25		650	7.6	0.06	0.04	0.36
26		820	8.2	0.08	0.05	0.36
27		1350	7.9	0.13	0.04	0.32
28	2.5gm. methionine + 2.2 gm. leucine	1130	7.5	0.17	0.10	0.60
29	2.5gm. methionine + 2.2 gm. leucine	1100	6.9	0.18	0.09	0.70
30		1570	9.8	0.17	0.08	0.37
31		1070	7.5	0.12	0.06	0.36
il 1		720	8.0	0.09	0.06	0.37
2	2.5gm. methionine + 1.75 gm. serine	1000	10. 0	0.13	0.11	0.67
3	2.5gm. methionine + 1.75 gm. serine	1170	10.6	0.19	0.14	0.72
4		1610	9.4	0.16	0.08	0.36
5		900	6.6	0.09	0.05	0.27
6		1050	8.8	0.10	0.07	0.42
7	2.5 gm. methionine + 1.5 gm. alanine	1070	10.7	0.15	0.12	0.82
8	2.5 gm. methionine + 1.5 gm. alanine	750	8.9	0.12	0.10	0.62
9		600	8.8	0.07	0.05	0.34
10		750	10.0	0.10	0.06	0.37
11		1160	8.5	0.12	0.07	0.34
12	2.5 gm. methionine + 1.25 gm. glycine	1160	8.8	0.16	0.13	0.62
13	2.5 gm. methionine + 1.25 gm. glycine	1760	10.5	0.24	0.13	0.82
14		630	7.3	0.11	0.05	0.37
15		600	8.7	0.11	0.05	0.31
16		620	9.1	0.08	0.05	0.34
24		1100	10.2	0.13	0.04	0.32
25		870	9.2	0.11	0.04	0.27
26		1200	8.8	0.13	0.05	0.31
27	1 gm. cystine	720	10.4	0.08	0.05	0.61
28		1450	10.0	0.16	0.06	0.38
29		1050	8.8	0.10	0.05	0.34
30	1.1 gm. homocystine	850	6.3	0.12	0.09	0.39
1		1150	8.4	0.13	0.07	0.33
2		1150	7.5	0.11	0.05	0.32
3	1.3 gm. serine	1020	8.5	0.14	0.07	0.28
4		1470	7.6	0.13	0.05	0.31
5		1780	9.9	0.15	0.08	0.44
6	1.3 gm. alanine	1130	7.3	0.14	0.05	0.31
7		920	8.1	0.10	0.07	0.35
8		560	7.2	0.07	0.04	0.30
9	1.25 gm. methionine	900	8.7	0.11	0.07	0.55
10		1000	8.9	0.12	0.08	0.54
11		750	7.5	0.09	0.07	0.27
12	1.3 gm. glycine	840	8.1	0.09	0.06	0.41
13		800	9.8	0.10	0.08	0.49
14		750	6.7	0.10	0.06	0.25
15	2.2 gm. leucine	1100	6.3	0.13	0.06	0.24

TABLE II.

EXTRA SULPHUR EXCRETED AFTER VARIOUS AMINO ACIDS.

Amino Acid Supplement	Date	Average N (gm)	Extra Sulphur excreted as Cystine gm.	Cystine p.c.	Methionine gm.	Methionine p.c.	Sulphate gm.	Sulphate p.c.
1 gm. Cystine	April 27-28	10.2	0	0	0.02	7	0.40	148
1.25 gm. Methionine	May 9-10	8.8	0.08	30	0.06	24	0.50	180.
1.1 gm. Homocystine	April 30-May 1	6.8	0.05	17	0.06	24	0.04	15
1.3 gm. Serine	May 3-4	8.0	0.04		0.01		- 0.05	
1.3 gm. Alanine	May 6-7	7.7	0.01		0		- 0.07	
1.3 gm. Glycine	May 12-13	9.0	0		0		0.35	
2.2 gm. Leucine	May 15	6.3	0.03		0		0.02	
5 gm. Methionine + 3.5 gm. Serine	April 2-4	10. 0	0.17	16	0.15	14	0.63	59
5 gm. Methionine + 4.4 gm. Leucine	March 28-30	8.0	0.25	23	0.14	13	0.64	59
5 gm. Methionine + 3.0 gm. Alanine	April 7-9	9.5	0.09	8	0.08	7	0.74	78
5 gm. Methionine + 2.5 gm. Glycine	April 12-14	8.8	0.18	17	0.11	10	0.74	78

FIGURE 1

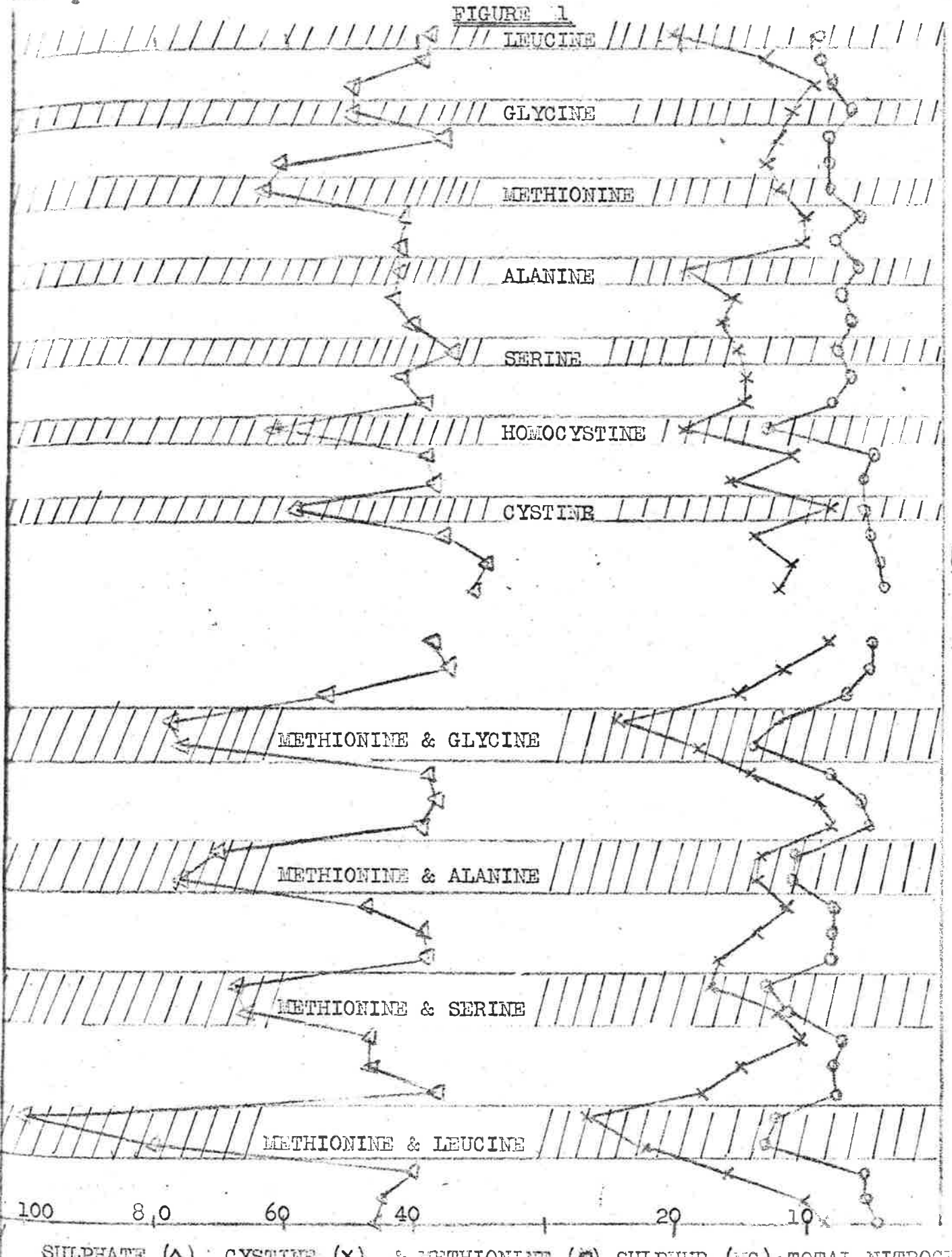

FIG. 1

SULPHATE (Δ), CYSTINE (×), & METHIONINE (⊙) SULPHUR (MG): TOTAL NITROGEN (G)

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by JOAN B. CLELAND

CYSTINE, METHIONINE AND SULPHATE EXCRETION IN CYSTINURIA

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Recent experiments have shown that there may be a considerable variation in the response of cystinuric individuals to the ingestion of sulphur-containing amino acids. All cystinurics so far recorded have shown an increased excretion of cystine when the protein intake was increased but not when extra cystine itself was ingested. Lewis, Brown and White (1936) and Brand, Cahill and Harris (1935) have both reported cases in which methionine and cysteine hydrochloride have increased the cystine excretion. Lough *et al.* (1941) found that one cystinuric woman excreted extra cystine after taking methionine only when the protein intake was low. A pre-adolescent boy examined by Andrews and Randall (1935) did not respond in this way to methionine. When, however, he was re-examined several years later (Andrews, Andrews and Runtenber, 1938; Andrews and Andrews, 1939) it was found that an increase in urinary cystine did follow the ingestion of this amino acid. More recently Hess and Sullivan (1942) have reported a case in which neither cysteine hydrochloride nor methionine affected the cystine excretion. Another cystinuric studied by them at the same time responded not only to methionine and cysteine, but also to alanine and, to a lesser extent, to glycine and glutamic acid.

Because of this variation in the response to different amino acids it seems advisable to examine as wide a range of subjects and conditions as possible. This paper gives the results obtained with a cystinuric woman.

EXPERIMENTAL DETAILS.

The subject of these studies was a young woman, 23 years of age, who in July, 1944, had her left kidney removed because of the presence of a cystine calculus in the renal pelvis. Specimens of urine collected a fortnight after the operation were acid in reaction and were loaded with cystine crystals. The patient was then given alkalis until the urine remained markedly alkaline, and this therapy was continued throughout the experiments reported here. It was found that when the pH fell below 7·6 crystals were invariably present.

Diet.

The patient was put on a meat-free diet which contained each day the same number of eggs and the same amount of milk but was unrestricted in cereals, fruit and vegetables. The total protein intake was approximately 0·9 gm. per kilo. As the patient was boarding it was found impossible to impose more rigid restrictions.

The amino acids, cystine, methionine and cysteine hydrochloride, were taken in gelatin capsules in such amounts that the intake each day corresponded in sulphur content to 2 gm. cystine. The l-cystine used on August 24th and 25th and the dl-methionine were made available through the courtesy of Mr. H. R. Marston. The cystine used on November 15th and 16th was isolated from sheep's wool and cysteine hydrochloride was obtained from part of the same preparation by reduction with tin and hydrochloric acid.

On those days when the protein was derived from a different source (October 25, 26, 31, November 1, 21 and 22) it was given in amounts which corresponded in nitrogen content to the eggs which it replaced, i.e. 14 gm. gelatin or 3 oz. lean veal replaced 2 eggs.

During the experiment beginning on November 21 the protein intake was increased by including one serve of meat (about 3½ oz.) in the usual diet, making the total protein intake about 1·2 gm. per kilo.

Method.

The 24 hour specimens of urine were preserved under toluene. The total nitrogen was determined by the usual modified Kjeldahl method and the total sulphate gravimetrically (Folin). A comparator was used for measuring the pH.

The Lugg modification of the Sullivan procedure (Lugg, 1933) was used to determine the cystine content of the urine after it had been standing for three days, but otherwise no effort was made to include the "bound cystine" which has been reported by Hess and Sullivan (1942) and Sullivan and Hess (1936) to be present in the urine of cystinurics. When cystine crystals were present the urine was centrifuged, the deposit dissolved in 3 p.c. HCl and the cystine content determined separately. Results given in the Tables include both the cystine present in solution and that obtained as crystals.

Methionine was determined by the method described by Albanese *et al.* (1944), except that as most of the 24 hour specimens of urine were of rather low volume the estimations were done directly on the acidified samples without previous concentration. The estimations were done in duplicate and gave consistent results. In view of the possible interference by cystine present in the urine of this patient a series of determinations was carried out on samples to which extra cystine had been added. It was concluded that cystine did not interfere with the determination of methionine in urine under the conditions of the experiment.

Unfortunately details of this method of estimating methionine were not available until 4 months after the urine samples had been collected. During this period the specimens, which had a pH of 7·6–8·0, and had had toluene added, were kept at 4° C. In view of the results there seems no reason to believe that there was any considerable loss of methionine during this period. The daily methionine excretion was found to be of the same order as that obtained from a number of normal individuals. Twenty-four estimations on urine from twelve adults gave results varying from 250 to 640 mg. methionine per day, the majority lying between 300 and 450 (compare Albanese *et al.*, 1944, who found in seven normal individuals a range of 247 to 494 mg. methionine excreted each day). Two specimens of normal urine allowed to stand under similar circumstances for 8 weeks showed no loss of methionine.

RESULTS AND DISCUSSION.

The results are shown in Tables 1, 2 and 3 and Fig. 1. The excretions of extra cystine, methionine and sulphate following the ingestion of certain amino acids and proteins were calculated for the two days on which they were fed and the following day. Normal values were obtained from the two, or where possible, three days preceding the test period.

Cystine.

When cystine was fed to the patient the effect was similar to that obtained with other cystinurics, that is, there was a rise in urinary sulphate during the following 24 hours but no increase in cystine. In fact, on both occasions, there was a slight fall in cystine excretion which was not accompanied by a corresponding fall in nitrogen excretion (Fig. 1). The per cent. of cystine recovered as sulphate was lower than that obtained by the majority of workers. The most probable explanation of this is that, the protein intake being unusually low, a high proportion of ingested cystine was used for tissue replacement. As others have found, the urinary sulphate excretion had returned to normal 24 hours after the ingestion of this amino acid.

Methionine.

The increase in urinary excretion of sulphate and of cystine which followed the ingestion of methionine was similar to the response obtained from the majority of cystinurics, though the per cent. recovery as cystine was higher and as sulphate was lower than usual Lough *et al.* (1941) and Lewis *et al.* (1936) who used similar doses of

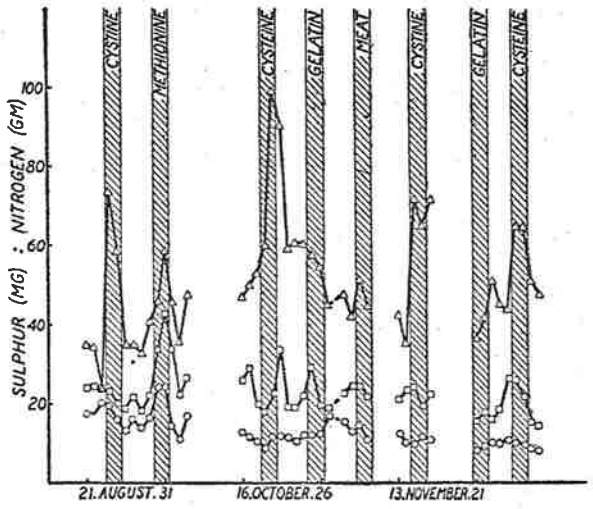

Fig 1. Ratio of urinary sulphate, cystine and methionine sulphur to total urinary nitrogen excreted each day.

△ Sulphate sulphur (mg.) : Total nitrogen (gm.).

□ Cystine sulphur (mg.) : Total nitrogen (gm.).

O Methionine sulphur (mg.) : Total nitrogen (gm.).

methionine found that less of it appeared as cystine when the protein intake was high (about 1·9 gm./kilo.) than when it was moderate (1·2 gm./kilo.). Brand, Cahill and Harris (1935) who studied a cystinuric on a lower protein diet (average nitrogen excretion 7·0 gm./day) recovered about 14 p.c. methionine as cystine. The large proportion of methionine obtained as cystine in this experiment can therefore be attributed largely to the small amount of protein consumed at this time (urinary N. 4·8 gm./day).

TABLE 1.

Influence of protein and of S-containing amino acids on urinary sulphur in cystinuria.

Date.	Substance fed.	Volume. ml.	Total N. gm.	Sulphur (gm.). Cystine.	Methionine.	Sulphate.
Aug. 21*		1,300	6·1	0·15	0·11	0·21
„ 22*		1,650	4·7	0·12	0·08	0·16
„ 23*		1,200	2·9	0·07	0·06	0·07
„ 24	2 gm. cystine	1,870	5·2	0·12	0·10	0·38
„ 25	„ „	1,200	3·7	0·07	0·06	0·22
„ 26		1,750	5·3	0·10	0·07	0·18
„ 27*		1,320	4·4	0·10	0·07	0·15
„ 28*		1,575	5·6	0·10	0·08	0·19
„ 29*		1,870	4·6	0·10	0·08	0·19
„ 30	2·5 gm. methionine	1,770	4·6	0·14	0·12	0·21
„ 31	„ „	1,980	6·2	0·27	0·15	0·37
Sept. 1		1,850	5·0	0·17	0·07	0·23
„ 2		1,100	4·2	0·09	0·05	0·10
„ 3		1,470	4·7	0·13	0·08	0·22
Oct. 16†		1,040	5·2	0·14	0·07	0·25
„ 17†		700	4·8	0·14	0·06	0·25
„ 18†		550	4·7	0·10	0·05	0·25
„ 19	2·7 gm. cysteine hydrochloride	600	5·4	0·10	0·05	0·33
„ 20	„ „ „	750	5·2	0·12	0·06	0·52
„ 21		1,050	5·4	0·18	0·06	0·50
„ 22†		720	5·4	0·10	0·06	0·32
„ 23†		1,200	6·2	0·12	0·07	0·37
„ 24†		610	4·6	0·10	0·06	0·28
„ 25	14 gm. gelatin replaced 2 eggs	1,090	5·4	0·16	0·07	0·31
„ 26	„ „ „ „	810	5·1	0·10	0·06	0·28
„ 27		810	5·2	0·10	0·09	0·23
„ 28		No specimen collected.				
„ 29†		1,140	5·7	0·16	0·09	0·27
„ 30†		700	5·3	0·13	0·07	0·22
„ 31	3 oz. veal replaced 2 eggs	670	5·0	0·13	0·08	0·26
Nov. 1	„ „ „ „	950	6·0	0·13	0·07	0·27
„ 13		1,300	7·0	0·14	0·09	0·31
„ 14		1,270	5·0	0·12	0·05	0·16
„ 15	2 gm. cystine	720	5·3	0·13	0·05	0·38
„ 16	„ „	730	5·0	0·10	0·06	0·33
„ 17		600	3·4	0·08	0·04	0·25
„ 21	14 gm. gelatin replaced 2 eggs	880	8·2	0·14	0·07	0·30
„ 22	„ „ „ „	1,100	8·5	0·16	0·08	0·35
„ 23‡		900	6·8	0·10	0·07	0·36
„ 24‡		800	7·4	0·14	0·07	0·33
„ 25‡		800	4·5	0·12	0·05	0·20
„ 26	2·7 gm. cysteine hydrochloride	1,350	10·0	0·26	0·10	0·65
„ 27	„ „ „	800	6·8	0·16	0·07	0·44
„ 28		1,230	9·7	0·15	0·08	0·50
„ 29‡		1,100	9·2	0·13	0·08	0·44

* These values used in calculating average values for low protein diet period 1. (Table 3.)
† These values used in calculating average values for low protein diet period 2. (Table 3.)
‡ These values used in calculating average values for medium protein diet. (Table 3.)

Recently experimental evidence of the way in which methionine is broken down to cysteine has been obtained. Binkley and du Vigneaud (1942) observed that when dl. homocysteine and dl. serine were shaken with rat liver slices l-cysteine was formed while Stetten (1942) has shown that cystine derived from rats fed with serine labelled with N^{15} had an unusually high proportion of the isotope. Since the inborn error in the metabolism of cystinuries is in handling cysteine the amount of extra urinary cystine derived from methionine would depend upon the availability of serine and methionine, the rate of demethylation and the rate of conversion of homocysteine to cysteine. When the protein intake is low the cystine excretion may be limited by lack of available serine and methionine, but when the protein intake is high the rate of conversion of homocysteine to cysteine may already be at its maximum so that the ingestion of extra methionine cannot increase cysteine production and this amino acid is disposed of by some other mechanism. This would mean that at high protein levels neither homocysteine nor methionine would produce extra cysteine (as was found by Lough and colleagues, 1939) and that equivalent amounts of methionine and homocysteine should give similar increases in the urinary cystine excretion at lower protein levels as was found by Brand, Cahill and Block (1935).

Information about the excretion of methionine by normal individuals is scanty. Values obtained throughout these experiments were of the same order as those found by Albanese *et al.* (1944) for normal individuals, and were within the range found for twelve adults whose urine was tested during these experiments. Andrews and Randall (1935) using a method involving the recovery of methyl iodide found that both a normal and a cystinuric boy excreted 20–40 mg. methionine sulphur per day and that in both cases the administration of 2·33 gm. dl-methionine caused an excretion of 10–15 p.c. of the ingested methionine. The recovery of methionine in these experiments lay within the same range, though the normal urinary methionine was higher. It cannot be assumed that the undetermined organic sulphur fraction as reported by most other workers is largely due to methionine, as the Benedict-Denis method of determining total sulphur (which is usually used) is known to give unreliable results when methionine is present (Masters, 1939).

As would be expected there was an immediate rise in methionine excretion after the administration of this amino acid, followed by a slower rise in cystine and sulphate excretion. This no doubt reflects the blood level of these constituents. Brown and Lewis (1941) found that in normal rabbits the ingestion of methionine was followed by a rise in plasma cystine and that the organic sulphur rose to a higher level for longer periods than after an equivalent amount of cystine, while the increase in sulphate content was greater and occurred more rapidly after cystine.

TABLE 2.

Extra sulphur excreted after cystine, methionine and cysteine hydrochloride.

Substance fed.	Amount fed. (gm.).	Date.	Average N. excretion (gm./day).	Extra sulphur excreted as Cystine. gm.	p.c.	Methionine. gm.	p.c.	Sulphate. gm.	p.c.	P.c. amino acid recovered.
Cystine	4	Aug. 24–26	4·8	—0·05	—5	—0·02	—2	0·34	32	32
„	„	Nov. 15–17	5·2	—0·09	—8	—0·06	—5	0·25	24	24
Methionine	5	Aug. 30–Sept. 1	4·8	0·28	26	0·11	10	0·28	26	62
Cysteine hydrochloride	5·4	Oct. 19–21	5·3	0·02	2	0	0	0·60	56	58
„	5·4	Nov. 26–28	8·8	0·21	20	0·06	5	0·70	66	91

Cysteine Hydrochloride.

In contrast to cystine the ingestion of cysteine hydrochloride was followed by a rise in cystine which accounted for 1 p.c. ingested compound when the protein intake was low, and 19 p.c. when the protein intake was raised to 1·2 gm./kilo. This variation could be attributed to the very low protein content of the diet during the earlier experiment assuming that at this level relatively more cysteine can be used as such. A higher proportion of the cysteine hydrochloride appeared as sulphate than occurred after either cystine or methionine, and more was excreted when the protein intake was raised. However, only in the latter case did the total recovery as cystine and sulphate approach values obtained by other workers (80–98 p.c.). This can also be attributed to greater utilization of cysteine as such when there is little protein in the diet.

An enzyme capable of converting cysteine to H_2S, pyruvic acid and ammonia has been isolated from the liver of rats (Laurence and Smythe, 1943) and mice (Binkley, 1943) which resembles and may be identical with that which produces pyruvic acid from serine. It is conceivable that in the cystinuric there is a deficiency of this enzyme so that excess cysteine accumu-

lates and appears in the urine as cystine. At the same time the failure of the enzyme to remove extra serine might be responsible for the preferential oxidation of methionine to cysteine.

In these experiments at a moderate level of protein intake (though not at a low level) the ingestion of cysteine hydrochloride caused a small rise in methionine excretion, but this was accompanied by an equivalent rise in urinary nitrogen.

Replacement of Eggs by Gelatin or Meat.

In order to reduce the concentration of cystine in the urine of cystinurics it is usual to advise the consumption of a diet low in protein, more particularly in animal protein which contains on the whole, rather more cystine and methionine than that of vegetable origin. It was therefore of interest to see whether the substitution of proteins containing different proportions of the sulphur-containing amino acids would affect the urinary concentration of cystine appreciably. The two eggs in the basal diet were replaced on two consecutive days by either gelatin or muscle meat containing an equivalent amount of protein. Assuming that these foodstuffs had the following amino-acid composition (Block and Bolling, 1944):

	Cystine.	Methionine.
Eggs	2·1 p.c.	4·0 p.c.
Meat	1·1 p.c.	3·3 p.c.
Gelatin	0·1 p.c.	0·8 p.c.

then, when meat replaced eggs, there would be a reduction in cystine intake of the order of 150 mg./day, and of methionine of 170 mg./day; and when gelatin replaced eggs the reduction would be about 230 mg. cystine and 340 mg. methionine.

An examination of the results shows that at this level of protein intake the use of protein with a lower content of sulphur-containing amino acids did not reduce either the cystine, methionine or sulphate excretion appreciably, and, in fact, there was a slight rise in cystine excretion after the gelatin. This suggests that either the protein intake was so low that the extra cystine and methionine which would be derived from the eggs (provided it was not taken in large amounts over a short period, as after test doses of the amino acids) was required and completely utilized by the tissues, or that cystine excretion is influenced more by the amount than the kind of protein consumed. Hess and Sullivan (1942), for example, found that in one cystinuric the ingestion of alanine and to a lesser extent of glycine and glutamic acid caused a rise in cystine excretion. Possibly a similar effect was obtained in this case so that a lower intake of S-containing amino acids was counteracted by the increased excretion caused by the glycine present in the gelatin. It should, however, be remembered that the expected difference would be very small if (as occurred when the pure amino acid was fed) only about 24 p.c. cystine and 26 p.c. methionine were recovered as sulphate, 26 p.c. methionine as cystine and 10 p.c. as methionine. The expected values for sulphur excretion obtained by calculation and those found by experiment are shown in Table 3. Both on theoretical and on practical grounds the restriction in the consumption of proteins rich in S-containing amino acids (at this level of protein intake) would be unwarranted in this cystinuric as a means of reducing the excretion of cystine.

TABLE 3.

Expected and observed differences in sulphur excretion with variations in protein intake.

			Av. S (gm./day) excreted as			Expected S (gm./day) excretion as		
Protein level.	Date.	Average N. excretion (gm./day).	Cystine.	Methionine.	Sulphate.	Cystine.	Methionine.	Sulphate.
Low (1)	Aug.*	4·8	0·11	0·08	0·16			
Low (2)	Oct.†	5·2	0·12	0·07	0·27			
Medium	Nov.‡	7·0	0·12	0·07	0·33	0·14	0·08	0·30
Gelatin replaced eggs	Oct. 25–26	5·2	0·12	0·07	0·28	0·10	0·06	0·23
" " "	Nov. 21–22	7·8	0·15	0·07	0·33	0·10	0·06	0·29
Meat replaced eggs	Oct 31–Nov. 1	5·5	0·13	0·07	0·27	0·11	0·07	0·25

*, †, ‡ See Table 1.

Affect of Different Levels of Protein Intake.

Throughout these experiments the nitrogen excretion was sometimes rather irregular and occasionally fell to values which were less than would be expected even from a fasting individual. In most cases this seems to have been due to a failure in the collection of an exact 24 hour specimen of urine as the reduction in nitrogen content was usually associated with a fall in the other constituents too, and low values were followed by ones rather higher than normal on the succeeding day. To overcome this difficulty the results have been expressed as cystine, methionine and sulphate sulphur per unit of nitrogen excreted (Fig. 1). This presentation of the results also shows the relative efficiency of the metabolism of the S-containing amino acids under different circumstances.

Although the amount of protein consumed remained the same, the nitrogen excretion during October and up to November 17 was higher than that during August and September. Not only did the total nitrogen increase, but the sulphate excretion was about 60 p.c. higher during the second period, while the methionine and cystine excretion remained about the same. A possible interpretation of this is that in August when the patient was still convalescing after the removal of one kidney, there was considerable retention of protein and S-containing amino acids (presumably chiefly cystine) for tissue repair.

When the protein intake was raised by including about 3½ oz. meat in the usual diet, an increase in cystine, methionine and sulphate excretion would be anticipated. In practice it was found that urinary sulphate was higher, and the cystine and methionine lower than would be expected (Table 3). Other investigators (Lewis and co-workers, 1936; Lough and co-workers, 1941) have drawn attention to the fact that less cystine is excreted after the ingestion of methionine at a high than at a moderate level of protein intake and attribute this to better utilization. The noticeable reduction in the ratio of urinary methionine to nitrogen and, to a smaller extent, of urinary cystine to nitrogen (Fig. 1) found when the protein intake was raised from 0·9 gm./kilo. to 1·2 gm./kilo. gives more conclusive evidence of this increased efficiency in the utilization of methionine.

SUMMARY.

Total nitrogen and cystine, methionine and sulphate sulphur have been determined in the urine of a cystinuric woman.

When cystine was fed no extra cystine or methionine was excreted, but there was a marked rise in urinary sulphate during the succeeding 24 hours.

When methionine was fed there was an immediate rise in methionine excretion followed by a slower rise in cystine and sulphate excretion which continued for two days.

When cysteine hydrochloride was fed the methionine excretion remained constant, but there was an increase in urinary cystine and sulphate which was more marked when the protein intake was raised from 0·9 to 1·2 gm./kilo.

The p.c. recovery of cystine, methionine and cysteine as sulphur in the urine was much less than in other cystinurics. This is believed to be due to the small amount of protein consumed during these experiments.

When the protein intake was increased from 0·9 to 1·2 gm./kilo there was a rise in the excretion of cystine, methionine and sulphate excretion. The proportion of cystine and methionine excreted per unit of nitrogen was less, and that of sulphate sulphur more at the higher than the lower level of protein intake.

Two levels of excretion were found when the patient was receiving 0·9 gm. protein/kilo. One level was determined shortly after the patient had undergone a severe surgical operation and showed a markedly lower excretion of nitrogen and sulphate sulphur.

When the two eggs in the basal diet were replaced by an equivalent amount of gelatin the expected fall in cystine, methionine and sulphate excretion did not take place.

The replacement of the eggs by meat was not accompanied by a fall in sulphur excretion.

The use of plant proteins in preference to those of animal origin as a means of reducing the cystine excretion in this patient is considered unwarranted when the protein consumption is low or moderate.

Possible interpretations of these results are discussed.

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CYSTINE, METHIONINE AND SULPHATE EXCRETION IN CYSTINURIA

by JOAN B. CLELAND

CYSTINE, METHIONINE AND SULPHATE EXCRETION IN CYSTINURIA

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A number of experiments have been carried out on the urinary excretion of cystinurics (Andrews *et al.*, 1938; Brand, Cahill and Harris, 1935; Cleland, 1945; Hess and Sullivan, 1942; Lewis *et al.*, 1936; Lough *et al.*, 1941). In all these cystinurics the excretion of cystine was about half a gram or more each day. Sullivan and Hess (1936) found that the cystine excretion of five normal individuals was about 100 mg. per day; and another normal individual examined by them (Hess and Sullivan, 1942), excreted about 150–200 mg. per day. This paper reports results obtained from a man who excreted about 250–350 mg. per day.

EXPERIMENTAL DETAILS.

History.

The subject of these studies was a man 65 years of age who was admitted to hospital on the 4th September, 1945, with a maculo-papular rash of unknown origin. His temperature was 103° F, and blood cultures and agglutinations were negative. On the 9th September he began to vomit and developed intractable hiccough. Urine examined on the following day was loaded with cystine crystals. While in hospital prior to this the urine was alkaline and fairly concentrated (S.G. 1,030–1,025) but the centrifuged deposit was not examined. On the 11th September he received sulphathiazole with alkalis and copious fluids, and this treatment was continued until the 17th September. The first 24-hour specimen of urine for analysis was collected on the 19th September.

Diet.

The patient's basal diet was such that on each day he ate 2 eggs, 1 serve (3 oz.) of meat, 1 pint of milk and an unlimited amount of cereals, fruit and vegetables. The total protein intake was about 1·2 gm. per kilo body weight.

The amino acids given for experimental purposes were taken in gelatine capsules with breakfast on the morning of the day indicated.

Cystine, methionine or cysteine hydrochloride was given in such amounts that the intake each day corresponded in sulphur content to 2 gm. cystine. The cystine was isolated from sheep's wool and the cysteine hydrochloride was prepared from the cystine by reduction with Sn and HCl. The methionine was made available through the courtesy of Mr. H. R. Marston.

Five gm. glycine (May and Baker) were given on two consecutive days (cf. Hess and Sullivan, 1942) but unfortunately only 3·2 gm. alanine (B.D.H.) were available and this was given in a single dose.

The two eggs in the basal diet were replaced on October 15th and 16th by 14 gm. gelatine and on October 31st and November 1st by 16 gm. casein, each of which contain an equivalent amount of protein. In addition to the basal diet, 14 gm. gelatine were eaten on October 19th and October 20th; 2 additional eggs on October 23rd and October 24th and 16 gm. casein on October 27th and October 28th. As this was not accompanied by a marked rise in urinary nitrogen there must have been a corresponding reduction in the consumption of vegetable proteins.

Methods.

The first specimen of urine collected was alkaline and the specific gravity 1,020. No cystine crystals could be detected, and during the whole series of experiments they were only occasionally present in the centrifuged deposit even though in some cases the pH fell below 6 and the urine

was very concentrated. Efforts made to obtain crystals by acidification, concentration and hydrolysis were not successful.

The daily excretions of nitrogen and of cystine, methionine and sulphate sulphur were determined under various conditions. In addition the content of cystine was determined in 24-hour urines from one daughter and two grand-children; and fasting urine specimens from his two other daughters and two grand-children were examined for the presence of cystine crystals.

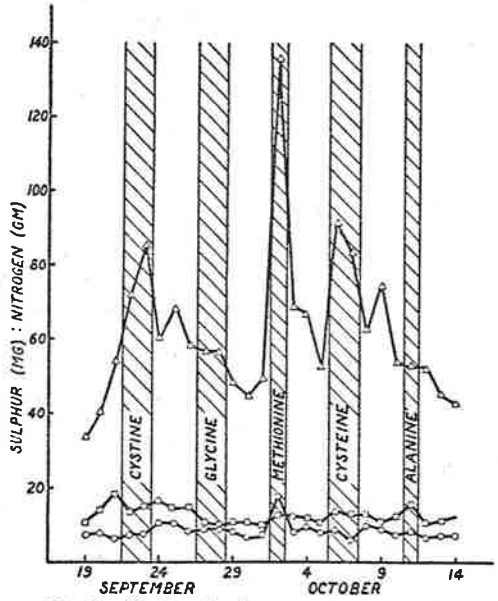

Fig. 1. Ratio of urinary sulphate, cystine and methionine sulphur to total urinary nitrogen excreted each day.

△ Sulphate sulphur (mg.): Total nitrogen (gm.).

□ Cystine sulphur (mg.): Total nitrogen (gm.).

○ Methionine sulphur (mg.): Total nitrogen (gm.).

The urine was collected under toluene and the methods used were those described in a previous paper (Cleland, 1945), except that a photo-electric colorimeter (photelometer-Cenco) was used for the final reading in the Lugg modification of the Sullivan procedure for estimating cystine (Lugg, 1933). Csonka *et al.* (1944), have studied the transmission curve of the red-coloured cystine complex and found that maximum absorption occurred at 505 mμ. At this point transmission through a solution of the unchanged naphthoquinone reagent was almost complete if the concentration was less than 0·3 p.c. In these experiments, using a filter which transmitted light between 450 mμ and 550 mμ and using 0·3 p.c. naphthoquinone reagent in place of the more concentrated 0·02 M solution used by Lugg, the blank solution containing only reagents gave 94 p.c. transmission. Results obtained by using the photelometer could be read more easily and rapidly than with a colorimeter, and gave consistent results.

RESULTS AND DISCUSSION.

Results are shown in Tables 1, 2 and 3 and Fig. 1. The excretions of extra cystine, methionine and sulphate following the ingestion of certain amino acids were calculated for the two days on which they were fed, and for the following day. Control values were obtained from the two preceding the test.

Values used for comparing the effect of different proteins were obtained by finding the average excretion for the two days on which the protein was fed and the following day. Results obtained on October 14th, 18th and 22nd were used to calculate the excretion on the basal diet.

As the total nitrogen excretion varied somewhat about a mean, the results have also been given as the ratio of sulphate, cystine and methionine S respectively per gm. of nitrogen excreted per day (Fig. 1 and Table 3).

Sulphur-containing Amino Acids.

As with other cystinurics the ingestion of cystine itself was not followed by an increase in cystine excretion, but there was a marked rise in urinary sulphate during the following 24 hours which amounted to 36 p.c. of the ingested amino acid.

The response of other cystinurics to methionine has been variable. In some cases there has been an increase in cystinuria (Brand, Cahill and Harris, 1935; Cleland, 1945; Hess and Sullivan, 1942; and Lewis *et al.*, 1936); in one case cystinuria increased only when the protein intake was low (Lough *et al.*, 1941), and in another methionine had no demonstrable effect (Hess and Sullivan, 1942). In the present case there was a slight rise in the ratio of cystine sulphur to total nitrogen excreted, and 2 p.c. of the test dose was recovered as cystine. These results are of the same order as those obtained by Hess and Sullivan (1942) for a normal individual and the difference is therefore insignificant. Unfortunately only 2·5 gm. methionine were available, and it is possible that more clear-cut results would have been obtained if more methionine had been given on the following day. However, there is no doubt that a large proportion of the methionine was oxidized completely, as the extra sulphate obtained would account for 66 p.c. of the ingested dose. Another 12 p.c. of the methionine was recovered as the unchanged amino acid, a value

similar to that obtained by Andrews and Randall (1935) and Cleland (1945). These results would indicate that methionine, except possibly in large doses, does not affect the degree of cystinuria in this subject on a moderate protein intake.

TABLE 1.

Influence of protein and S-containing amino acids on urinary sulphur in cystinuria.

Date.	Substance fed.	Volume. ml.	Total N. gm.	Sulphur (gm.). Cystine.	Methionine.	Sulphate.
Sept. 19		680	7·1	0·08	0·06	0·24
20		1,310	8·3	0·12	0·07	0·33
21		960	3·2	0·06	0·02	0·17
22	2 gm. cystine	880	5·2	0·07	0·04	0·37
23	2 „ „	970	4·5	0·07	0·03	0·38
24		1,300	6·2	0·10	0·07	0·37
25		730	3·9	0·06	0·04	0·27
26		940	4·5	0·07	0·04	0·26
27	5 gm. glycine	910	6·1	0·07	0·06	0·34
28	5 „ „	910	7·9	0·08	0·08	0·44
29		710	4·2	0·05	0·04	0·20
30		1,330	6·5	0·07	0·04	0·30
Oct. 1		1,520	7·2	0·07	0·05	0·37
2	2·5 gm. methionine	1,430	6·4	0·08	0·12	0·66
3		970	5·4	0·07	0·05	0·37
4		680	5·9	0·07	0·06	0·40
5		780	6·8	0·08	0·05	0·36
6	2·7 gm. cysteine hydrochloride	770	5·7	0·08	0·05	0·52
7	2·7 gm. cysteine hydrochloride	1,630	9·3	0·12	0·06	0·78
8		1,010	5·7	0·08	0·06	0·35
9		520*	3·8	0·04	0·04	0·29
10		1,230	6·7	0·08	0·05	0·36
11	3·2 gm. alanine	1,370	6·6	0·11	0·06	0·35
12		1,150	8·0	0·09	0·05	0·43
13		1,210	8·2	0·09	0·06	0·37
14		1,220	7·0	0·09	0·05	0·30
15	14 gm. gelatine replaced 2 eggs in basal diet	1,360	6·9	0·10	0·06	0·33
16	14 gm. gelatine replaced 2 eggs in basal diet	1,480	8·4	0·11	0·06	0·50
17		1,100	6·8	0·09	0·06	0·47
18		850	7·3	0·08	0·06	0·46
19	14 gm. gelatine in addition to basal diet	850	7·8	0·07	0·05	0·41
20	14 gm. gelatine in addition to basal diet	850	8·3	0·09	0·06	0·52
21		1,130	7·6	0·10	0·06	0·41
22		750	6·2	0·06	0·06	0·42
23	2 eggs in addition to basal diet	1,250	6·7	0·09	0·07	0·51
24	2 eggs in addition to basal diet	1,850	8·2	0·11	0·06	0·51
25		1,000	6·8	0·07	0·05	0·50
26		1,360	9·5	0·09	0·05	0·55
27	16 gm. casein in addition to basal diet	1,130	7·5	0·08	0·06	0·45
28	16 gm. casein in addition to basal diet	1,460	8·5	0·11	0·06	0·51
29		1,140	8·2	0·11	0·05	0·68
30		1,650	8·3	0·14	0·04	0·44
31	16 gm. casein replaced 2 eggs in basal diet	1,350	7·4	0·14	0·08	0·65
Nov. 1	16 gm. casein replaced 2 eggs in basal diet	700	5·9	0·11	0·06	0·72
2		1,000	6·7	0·14	0·07	0·76
3		1,080	9·1	0·11	0·06	0·40

* Specimen incomplete.

TABLE 2.

Extra sulphur excreted after various amino acids.

Amino-acid fed.	Amount. (gm.).	Average nitrogen. (gm.).	Extra sulphur excreted as cystine. gm.	P.c.	methionine. gm.	P.c.	sulphate. gm.	P.c.	Amino-acid recovered. P.c.
Cystine	4	5·3	0	—	0	—	0·38	36	36
Glycine	10	6·1	0		0·05		0·19		
Methionine	2·5	5·9	0·01	2	0·07	12	0·35	66	80
Cysteine hydrochloride	5·4	6·9	0·05	5	0	—	0·52	49	54
Alanine	3·2	7·3	0·02		0·01		0·06		

Cystinuria is regarded as being due to a defect in cysteine metabolism, and with one exception (Hess and Sullivan, 1942), cysteine hydrochloride has caused a definite increase in cystine excretion in those cystinurics studied. In this case the rise in cystinuria after taking cysteine hydrochloride amounted to only 5 p.c. of the ingested acid, and as this increase in excretion was accompanied by a corresponding rise in urinary nitrogen, it might be due to an increase in metabolism as a whole rather than to a specific increase in cystine. The amount of extra cystine recovered after feeding with cysteine hydrochloride was so small that it is unlikely that this man has any marked inability to handle it. The fact that the sulphate excretion (49 p.c.) was higher than that obtained after an equivalent amount of cystine (36 p.c.) suggests that oxidation to sulphate is carried out more readily than with cystine itself.

Glycine and Alanine.

Hess and Sullivan (1942) gave glycine and alanine to two cystinurics and one normal subject, and found that in one of the cystinurics there was a marked increase in cystinuria following alanine, and a smaller one after glycine. The other cystinuric and the normal individual were not affected. When 5 gm. glycine were given to the present subject there was a marked rise in urinary sulphate and methionine, but no alteration in cystine excretion. When these values are compared with the concurrent nitrogen excretion, however, it is found that there was an equivalent rise in urinary nitrogen, or, in other words, the increase was due to an increase in protein metabolism as a whole and can probably be attributed to the specific dynamic action of the glycine. There seems no reasonable explanation of why the cystine excretion was not also raised, unless the presence of glycine alters the direction of methionine metabolism or increases the demand for cystine.

When alanine was given there was a definite increase in urinary cystine and sulphate but no alteration in the methionine. The rise in sulphate was accompanied by a proportional rise in nitrogen, but the cystine excretion per unit of nitrogen was higher after alanine than after any of the other amino acids tried, and more extra cystine was obtained after 3·2 gm. alanine than from 2·5 gm. methionine, though not as much as was obtained after a total of 5·4 gm. cysteine hydrochloride given on two consecutive days. This specific effect of alanine is difficult to explain. The recent work of Binkley and du Vigneaud (1942) and Stetten (1942) has shown that methionine is first demethylated to homocysteine which reacts with serine to form cysteine. It is possible that if alanine is the source of serine and if serine were a limiting factor in the rate of this reaction, then alanine might cause an increase in cysteine production. This does not seem very likely. Fromageot *et al.* (1941), working with liver from dogs, came to the conclusion that the following reaction occurred:

$$3 \text{ cysteine} \rightleftharpoons \text{cystine} + H_2S + \text{alanine}.$$

Smythe (1942) was unable to confirm the equation, and his results were more in accord with:

$$\text{cysteine} \rightleftharpoons \text{pyruvic acid} + H_2S + NH_3.$$

However, he (Smythe, 1945) considers that the production of some alanine may also occur. Smythe and Halliday (1942), too, have shown that when H_2S containing radioactive sulphur is included with the cysteine and the enzyme preparation some of the tagged sulphur is found in the cysteine, indicating that the reaction is reversible. If so, the presence of large amounts of alanine might not only limit the removal of cysteine but actually increase its production.

Variations of Protein Intake.

If methionine and cysteine are the source of urinary cystine then it would be expected that an increase in protein intake, or of the proportion of those proteins rich in methionine, would be followed by an increase in cystine excretion. The protein intake was therefore varied by substituting gelatine (poor in cystine and methionine) or casein (poor in cystine) for eggs (rich in both cystine and methionine) in amounts which contained approximately the same quantity of protein. A higher intake of animal protein (and incidentally of cystine and methionine) was

obtained by incorporating an extra 2 eggs in the basal diet. This did not result in any appreciable increase in urinary nitrogen and it can therefore be assumed that the eggs replaced some of the vegetable protein.

TABLE 3.

Effect of different proteins on excretion of S-containing amino acids.

Date.	Added protein.	Average urinary N.	Average daily increase in diet of cystine. (gm.).	Average daily increase in diet of methionine. (gm.).	Average daily excretion of cystine. (gm.).	Average daily excretion of methionine. (gm.).	Average excretion per gm. nitrogen of cystine S. (mg.).	Average excretion per gm. nitrogen of methionine S. (mg.).
Oct. 15–18	14 gm. gelatine	7·4	0·01	0·11	0·38	0·28	13·5	8·0
Oct. 31–Nov. 1	16 gm. casein	6·7	0·06	0·56	0·47	0·33	18·8	10·4
Oct. 14, 18, 22	2 eggs	6·9	0·27	0·59	0·28	0·26	11·2	8·3
Oct.19–21	2 eggs + 14 gm. gelatine	7·9	0·28	0·70	0·31	0·26	10·5	7·0
Oct. 27–29	2 eggs + 16 gm. casein	8·1	0·33	1·15	0·38	0·26	12·6	7·0
Oct. 23–25	4 eggs	7·2	0·54	1·18	0·34	0·28	12·5	8·3

The estimated differences in cystine and methionine content of the diet during the different periods can be seen in Table 3. These were based on the amino-acid content of proteins given by Block and Bolling (1944). An examination of the same Table shows that the lowest values were obtained for both cystine and methionine excretion when the basal diet—2 eggs, one serve of meat and 1 pint of milk, with cereals and fruit and vegetables—was consumed. The addition of gelatine to this was not followed by any appreciable alteration in the amino-acid excretion but, as would be expected, the cystine and methionine excretion per gram of nitrogen was reduced, the rise in urinary nitrogen being attributed to the specific dynamic action of glycine. On the other hand, when the cystine and methionine content of the diet was reduced by substituting gelatine for eggs, the excretion of cystine was greater than and the methionine the same as when 4 eggs (which contain about 1 gm. more methionine and ½ gm. more cystine) were included in the diet, whether the results were determined as extra amino-acid or amino-acid per gm. of nitrogen excreted. Again, when 2 of the 4 eggs were replaced by 16 gm. casein, the methionine excretion was a little lower but the cystine excretion higher, although the methionine intake was unaltered and the cystine intake reduced.

Highest values for methionine and cystine excretion (either as total excretion or per gm. of nitrogen) were obtained when the diet contained 16 gm. casein but no eggs, or, in other words, about 0·6 gm. of methionine but little cystine.

These results show that although cysteine and methionine may be the source of urinary cystine, the amount excreted is by no means proportional to the intake of these amino-acids. Lewis *et al.* (1936), and Lough *et al.* (1941), have already shown that in some cystinurics less cystine was excreted after a given dose of methionine when the protein intake was high than when it was moderate. Cleland (1945) found in a cystinuric who showed a marked response to both methionine and cysteine hydrochloride that alterations in the intake of S-containing amino-acids by varying the type of protein consumed or the total protein intake were not accompanied by equivalent alterations in cystine and methionine excretion.

From these figures it would appear that eggs contain some factor which depresses cystinuria in this individual. Probably its action is on methionine metabolism, for cystine excretion was greatest when in the absence of eggs the diet contained appreciable amounts of methionine. It is possible that cystine itself is responsible for some of this effect, for although ingested cystine did not alter the excretion of cystine, there was a tendency for the ratio of urinary cystine to nitrogen to fall (Fig. 1). It should, however, be remembered that whereas gelatine and casein are almost pure protein, eggs contain appreciable amounts of fat and other substances, some of which (e.g. choline or some of the vitamins) may be involved in methionine metabolism.

It has been suggested, (Cleland, 1945), that a high serine intake may prevent normal cysteine destruction while on a moderate or low protein diet cysteine production from methionine may be limited by the serine content of the diet. A rough estimation of the serine content of gelatine and casein, based on figures obtained by Stokes and Gunness (1945), shows that 14 gm. gelatine contain about 40 mg. serine, and 16 gm. casein about 100 mg. serine. The serine content of eggs has not been determined. If, then, serine were the limiting factor, only about 0·10 gm. cystine could be derived from the 560 mg. of methionine in the casein used. In actual practice the difference in the cystine excretion obtained when gelatine and casein were fed in the absence of eggs amounted to about 0·09

gm. Similarly one would expect that if methionine oxidation to cysteine were blocked by lack of serine more methionine would appear in the urine. Results obtained using casein would fit in with this hypothesis. When, however, eggs were included in the basal diet, the methionine excretion fell although the intake increased. This could be explained by assuming that eggs contain appreciable amounts of serine. On the other hand, there was no concurrent increase in cystine excretion which should have occurred if serine were the only factor involved. It can therefore be assumed that if serine is a factor which influences the cystine excretion in this cystinuric, it is not the only one other than the cystine and methionine content of the diet which is involved.

As the results obtained from this cystinuric differ quite markedly from those found in the majority of cases, they suggest the possibility that the two types may differ in aetiology. There seems no doubt that in most cystinurics there is an inherent inability to utilize cysteine normally, but in this case the evidence does not justify a similar conclusion. It is, of course, quite possible that the man has only a very mild degree of cystinuria and that he is able to utilize cysteine under all but exceptional circumstances, as, for example, when he became seriously ill and dehydrated. Another, and equally plausible explanation is that the condition is due not to a metabolic defect but to an abnormally low renal threshold. In this respect it is interesting to note that of his three relations examined, two—a daughter and grand-daughter (aged 12) excreted relatively large amounts of cystine (160 mg. and 140 mg. per day respectively) though these are still considered to be within the normal range. The other grand-daughter excreted about 60 mg. per day. It was impossible to examine 24-hour specimens of urine from other members of the family, but a rough colorimetric test on single specimens of urine showed that little cystine was present. No crystals of cystine were found in any of the specimens examined.

SUMMARY.

Total nitrogen and cystine, methionine and sulphate sulphur have been determined in the urine of a mild cystinuric who excreted about 250–350 mg. cystine per day.

When cystine, methionine or cysteine hydrochloride was taken by mouth there was a rise in urinary sulphate but no significant increase in cystine excretion. Twelve p.c. of ingested methionine could be recovered unchanged in the urine.

Glycine increased urinary nitrogen, methionine and sulphate in corresponding amounts, but did not affect the cystine excretion.

Alanine caused an equivalent rise in methionine, sulphate and total nitrogen, but the increase in cystine was relatively greater.

Cystine and methionine excretion were least when the basal diet, consisting of two eggs, one serve of meat, one pint of milk, cereals, fruit and vegetables was consumed; and greatest when 16 gm. casein replaced the two eggs in the basal diet. Values intermediate between these two extremes were obtained when the protein supplement to the basal diet (arranged in order of increasing cystine excretion) was 14 gm. gelatine; two additional eggs; 16 gm. casein. When 14 gm. gelatine replaced the two eggs in the basal diet, the results were of the same order as those obtained with the basal diet supplemented with 16 gm. casein.

The urine of two of the three relatives examined contained more than the average content of cystine, though the values still lay within the normal range.

Possible interpretations of these results are discussed.

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SOME OBSERVATIONS

ON

THE TOXICITY AND DETOXICATION OF CINEOLE

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THE TOXICITY AND DETOXICATION OF CINEOLE

INTRODUCTION.

Investigations on the relative toxicity of cineole are of interest because this terpene occurs in most of the essential oils of those eucalypts which form a large part of, and in the case of the koala bear the only, food of some Australian fauna. In those leaves most favoured by the koala about 1% of the leaf weight consists of essential oils of which cineole is the major constituent.

The only mention made of the destruction of cineole derivatives in the animal body is the recovery of β-terpineol sulphate from the urine of dogs given keto-cineole (Mascherpa, 1930). The detoxication of open chain terpenes has been studied by Kuhn et al. (1936), who isolated dimethylhexadiene dicarboxylic acid after feeding geraniol, citral and geranic acid to rabbits. Asahini and Ishidati (1935), showed that the administration of camphor to animals also resulted in the formation of a carboxylic acid, in this case by the oxidation of one of the central methyl groups. Other products (hydroxy derivatives) of camphor were excreted in conjugation with glucuronic acid. Harvey (1942) found that sheep formed cumic acid from cymene; phellandric acid, cymene and carvotanacetone from α-phellandrene; and thymol and carvotanacetone from piperitone.

EXPERIMENTAL METHODS.

i. Administration and Toxicity of Cineole.

Rabbits, rats, guinea pigs and opossums (Trichosurus vulpecula) were fed by means of a stomach tube with 0.5 gm. cineole per kilo

body weight. Doses were given at weekly intervals and were increased on each occasion by 0.1 gm. per kilo for guinea pigs or 0.2 gm. per kilo for the other animals until all had been killed. L.D. 50 was obtained by assuming that all animals which were killed by one dose would also be killed by all larger doses. The results are recorded in Table 1.

In addition 64 opossums each received a single dose of cineole ranging from 0.9 to 3 gm. per kilo. The death rate in these cases is recorded in Table 11.

ii. Collection of Urine.

The animals were kept in metabolism cages and 24-hour specimens of urine from these and other animals which had received cineole were collected without preservative for two days following the administration of the cineole. In certain cases urine specimens were collected for a week after the administration of the drug.

iii. Estimation of Glucuronic Acid.

A small quantity of urine was used for the determination of the glucuronic acid content by the method of Maughan et al. (1938), except that the colour produced with the naphthoresorcinol reagent was compared with that obtained from a standard solution of glucuronic acid containing 20 mg. per 100 cc. When necessary the urine was first diluted.

A rough estimation of the reducing properties of the urine was also made using Cole's ferricyanide method (Cole, 1933).

Results are shown in Tables 111 and 1V.

iv. Isolation of Hydroxy-Cineole.

The urine was distilled and the alkaline distillate was extracted with ether which was then allowed to evaporate. The treacle-coloured residue was distilled under reduced pressure (using a water-pump) and several fractions were collected. When doses of cineole exceeding 1 gm. per kilo were given to opossums, and more particularly with the larger doses, the first fraction collected from the ether extract yielded a clear viscid liquid which readily crystallised. Most of the oily matter was removed by placing the crystalline material on a porous porcelain plate and the remaining solid was recrystallised from petroleum ether from which it formed thin white needles. Two recrystallisations were necessary before the melting point remained constant at 69°C. The material was readily soluble in alcohol, ether, acetone and chloroform; and an 8% solution in acetone was optically inactive.

A few crystals were dissolved in alcohol, and to 2 cc. of this solution 0.2 cc. 1% p-dimethylaminobenzaldehyde in 30% alcohol was added. On the addition of 1 cc. conc. sulphuric or hydrochloric acid a purple ring developed at the junction of the two layers and on gentle shaking the upper layer became deep blue. When benzaldehyde was used as the reagent a purple colour with a red fluorescence was obtained, while with vanillin on the addition of conc. acid the initial green colour became purple. The first of these reactions - the development of a purple ring with p-dimethylaminobenzaldehyde and conc. acid - was used to detect urine containing hydroxy-cineole in reasonable amounts for extraction.

A microchemical analysis of the crystalline material was made by Dr. Berger and Mr. John Mills of the Chemistry Department of the Universityy of Adelaide. I am grateful to them for the following information:-

Elementry Constitution

	Carbon	Hydrogen
Material supplied	70.60%	10.76%
Sublimed material	70.63%	10.62%
Theoretical for $C_{10}H_{18}O_2$	70.53%	10.66%

The material reacted with p-nitrobenzoyl chloride to form a monoester of M.P. 107°C. which was found to contain 64.10% carbon and 6.76% hydrogen. Theoretical requiremnts for a monoester of hydroxycineole are 63.92% carbon and 6.63% hydrogen. It is therefore assumed that the substance isolated was hydroxy-cineole.

Oxidation with $KMnO_4$ in acetone gave oily products from which no crystalline 2-4 dinitrophenylhydrazone derivatives could be obtained.

The original crystalline product had a fairly clear melting point but on recrystallisation it melted over the range of 67-79°C., so the melting point was taken between glass cover slips on a hot plate in such a way that as part melted it could drain away from the crystals. Melting started at 64°C., and most of the material melted at 67-69 C°., but some large crystals were left unmelted at 82°C. This indicated that the supposedly pure material melting at 69°C. was a mixture. (It is possible that only one isomer was synthesised in the body, and that others were derived

from it during the isolation and recrystallisation).

Since the material was rather too soluble for easy recrystallisation and appeared to be fairly volatile, fractional sublimation was carried out. At intervals the process was interrupted and the sublimate scraped off. Four fractions were obtained:-

a. Coarse prisms. M.P. 64-67°C. About 25% of total

b. Fine needles. M.P. mainly 64-66°, but some up to 80°C. About 30% of total.

c. Large needles. M.P. 64-92°C (indefinite). About 25% of total

d. Long needles. M.P. 93-97°C. About 15% of total.

The material was then systematically resublimed, the more volatile fractions being sublimed first and the less volatile fractions added gradually as the process was continued. Even after the third cycle of resublimation the separation was incomplete, though the more volatile fractions were reaching a constant M.P. as likewise were the less volatile fractions. From about 1.8 gm. of material the final result was :-

a. 700 mg. fine needles; M.P. 96-98°C.

b. 670 mg. needles; M.P. 70-90°C.

c. 420 mg. more granular; M.P. 63-66°C., traces up to 70°C.

Losses were about 3 %.

An attempted recrystallisation of one of the intermediate fractions from X 60 solvent was not very encouraging; losses were large and the M.P. was very little alterred.

The only material obtained from an attempt to form a phenylurethane derivative from some of the higher-melting fraction was

diphenyl urea. The remaining resinous material had not crystallised after many months.

Identical methods were used in an attempt to isolate a similar compound from the urine of rabbits which had been given similar doses of cineole. A colourless oily liquid distilling at about the same temperature as the fraction from opossum urine which contained hydroxy-cineole was obtained, but despite redistillation and the use of seed crystals no crystallisation occurred. The oily fraction from rabbits gave a much weaker test with p-dimethylaminobenzaldehyde than did a similar fraction from opossums.

The urine from rats fed with cineole in similar doses also gave an oily fraction distilling at about the same temperature. This gave only a faint purple ring with p-dimethylaminobenzaldehyde.

The oily products obtained from the urine of koala bears (living exclusively on E. elaeophora) by a similar procedure gave a negative test with p-dimethylaminobenzaldehyde.

Other fractions obtained by the distillation of the ether extract of urine from opossums, rabbits and rats did not yield crystalline products and efforts to purify and identify them have not yet been carried out.

v. Detection of Other Products of Detoxication.

The following methods were used in an attempt to isolate some of the products of cineole detoxication:-

a. Precipitaion by half saturation with ammonium sulphate as used by Quick (1924), for the isolation of menthol glucuronate.

b. Precipitaion with zinc sulphate as used for the isolation of

borneol glucuronate. (Quick, 1927).

c. Precipitation with baryta water and lead acetate as used by Kuhn et al. (1936), for the isolation of terpene derivatives.

No detoxication products were obtained by any of these methods

DISCUSSION.

The Toxicity of Cineole.

Symptoms of cineole poisoning when they occurred usually appeared in 2-3 hours, though sometimes in rats they were noticeable within half an hour. They consisted of an ataxic gait (except in opossums which were asleep during the day) and shivering followed by circulatory and (more particularly) respiratory failure. In guinea pigs death from respiratory failure usually occurred within five hours, and in the other animals in 5-10 hours, though an occassional rabbit or opossum remained comatose for more than 24, and in one opossum for 72 hours before death supervened.

Macroscopically the dead animals showed only extensive congestion and occassional haemorrhages in the lungs. No pathological lesions were observed in sections from kidney, liver and lung tissues from a guinea pig and an opossum which had each been receiving cineole twice a week for several weeks (the opossum had received 40 gm. cineole in four weeks).

MacPherson (1925) states that cineole affects the nervous system, and the symptoms noted by Barker et al. (1918) in dogs and cats poisoned with cineole, and the results obtained in the present experiments with rabbits, rats, opossums and guinea pigs are in

agreement with this. Congestion of the lungs is also a common finding and has been reported in two cases of death from eucalyptus poisoning in humans (Foggie, 1911).

The figure of 1.68 gm. cineole per kilo given by Brownlee for the L.D.50 for rats was lower than the results reported for the same species in these experiments. On the other hand Barker et al. (1918) state that Bruning found that guinea pigs could withstand doses of 0.9 gm. eucalyptus oil (equivalent to 0.65 gm. cineole) a figure which is a fraction lower than the M.L.D. obtained for guinea pigs in the present experiments.

From a comparison of the M.L.D. and the L.D.50 for the animals used in these experiments it can be seen that the opossum appears to be just as susceptible to large doses of cineole as rabbits and rats, though less so than guinea pigs. When the L.D.50 is expressed as gm. cineole per 2/3 power of body weight the similarity in the first three groups of animals is even more striking.

A rough estimate of the quantity of cineole consumed by the koala bear (Phascolarctos cinereus) was made from the average weight (56 gm. per kilo) of eucalyptus leaves (E. elaeophora) consumed each day by several koalas. The cineole content of the leaves was obtained from the tables given by Baker and Smith (1902). The average daily intake of cineole was found to be about 0.2 gm. per kilo - roughly one quarter of the M.L.D. for guinea pigs or one sixth the M.L.D. for opossums.

Brownlee (1940) found that daily doses of 0.5 gm. cineole per kilo when given to rats resulted in death of the three animals

used within 19 days. In these experiments, too, there was evidence of the cumulative effects of the poison since in some animals the same dose produced symptoms of toxicity only on the third or fourth successive day of feeding. This cumulative effect was only noticeable with doses exceeding 1 gm. per kilo with the larger animals - that is a dose more than five times the average cineole consumption of the koala. It should also be remembered that the essential oils eaten by the koala are taken with other food , and the total intake is distributed over a period of several hours, whereas with the experimental animals cineole was given in a single large dose and was not diluted with other food material.

An interval of a week between doses appeared to be adequate for the complete recovery of experimental animals from cineole intoxication. Glucuronate excretion fell to within normal limits in four days, and the toxicity of cineole to opossums was no less when it was given in single doses (TableII) than when it was administered once a week in gradually increasing amounts. (Table 1).

Conjugation.

An examination of Tables 3 and 4 shows that in opossums and rabbits the ingestion of cineole was accompanied by a rise in the excretion of glucuronic acid and reducing bodies. The presence of the latter suggests either that the glucuronic acid derivative is ~~either~~ readily hydrolysed or that conjugation does not involve the potential aldehyde group. In both species with moderately high doses of cineole about half the ingested terpene could be accounted for

as increased glucuronic acid if it is assumed that one molecule of glucuronic acid is equivalent to one of cineole. Large doses (over 3 gm. per kilo.) were associated with a relatively greater excretion of glucuronic acid, in one opossum accounting for 80% of the cineole This suggests that conjugation with glucuronic acid is secondary to some other mechanism, possibly oxidation, the extent of which is limited so that with large doses of cineole a greater percentage must be combined with glucuronic acid.

When guinea pigs received cineole a much smaller increase in glucuronate was obtained and this accounted for only 14% of the cineole. In this respect it is of interest to note that the M.L.D. of cineole for guinea pigs was only half that for rabbits, rats or opossums.

Glucuronic acid conjugation would necessitate the introduction of some polar group into the cineole molecule, and, in the case of the opossum, hydroxy-cineole was actually isolated. The same compound could not, however, be isolated from the urine of rabbits or rats, but it is possible that in these animals other isomers of hydroxy-cineole were formed. By analogy with camphor and open chain terpenes the formation of some carboxylic acid would also be expected.

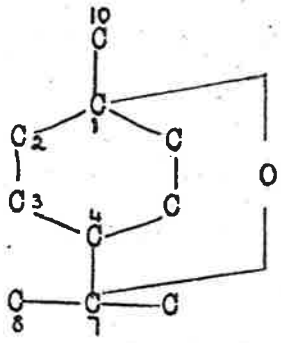
Mascherpa (1930) obtained β-terpineol sulphate from dogs which had been given 2-keto-cineole. If such a compound were formed from cineole it seems likely that in many animals it would be excreted as the glucuronate rather than the sulphate. Ketones have been known to be formed from other terpenes in the animal body

(cf. carvotanacetone from α-phellandrene as described by Harvey, 1942), and keto-cineole production may well be one step in the normal ~~production~~ metabolism of cineole. Cineole and keto-cineole have similar physiological properties so that the toxicity of the former could possibly be attributed to the formation of the latter.

Oxidation.

The microchemical analysis of the crystalline compound isolated from the urine of opossums after the ingestion of cineole showed that it contained one more oxygen atom than cineole and that this was present in the form of an hydroxy group. Several isomers were partially separated but it is impossible to say whether these were all originally excreted in the urine or whether some were formed during the isolation and recrystallisation of the compound.

By analogy with other terpenes oxidation would be expected to occur at C_8



Such a compound would have an assymetric carbon atom and would therefore be expected to yield an optically active compound from biological material. This was not obtained. On the other hand oxidation at C_{10} would yield an optically inactive isomer. For chemical reasons substitution at C_4 is most unlikely

to occur.

Although substitution in the nucleus is not very common in the biological detoxication of the terpenes, some of the hydroxy-cineole isolated resembled quite closely in the few properties studied the cis and trans forms of 2-hydroxy-cineole as given by Gandini (1937). One of the isomers isolated by him and believed to be the cis form melted at 80°C, sublimed readily and gave a purple colour with vanillin and HCl. A 2% solution in acetone was optically inactive. The other isomer (trans ?) which occurred in small amounts melted at 108°C. Separation of the two isomers was only obtained by isolating suitable derivatives and obtaining the original hydroxy-cineole by decomposition of the product. It therefore seems likely that the two less volatile fractions obtained from the urine were the cis and trans forms of 2-hydroxy-cineole.

Unfortunately, owing to the failure to form suitable crystalline derivatives, it has been impossible to state at which point the hydroxy group occurs in the hydroxy-cineole isolated. Three isomers appear to have been obtained and two of these resemble the cis and trans forms of 2-keto-cineole. No clue to the structure of the third isomer (M.P. 63-66) has been obtained, but by analogy with other terpenes oxidation at C_8 would be expected.

SUMMARY.

The relative toxicity of cineole was determined in rats, rabbits, opossums and guinea pigs, and the L.D. 50 was found to be almost the same in the first three species (2.5 gm. per 2/3 power of body weight in kilos), while for guinea pigs it was only half of this (1.2 gm). M.L.D. was 1.6, 1.2, 1.2 and 0.7 gm. per kilo respectively. With larger doses there was evidence of cumulative poisoning when cineole was given on consecutive days.

There was no evidence of specific resistance to cineole poisoning in opossums, but the M.L.D. was about six times the probable intake of cineole by the koala bear which lives exclusively on eucalyptus leaves. It therefore seems unlikely that specific resistance to cineole would be necessary in these animals.

Ingestion of cineole led to an increase in the excretion of glucuronic acid in rabbits, opossums and, to a small extent, in guinea pigs.

Hydroxy-cineole was isolated from the urine of opossums given cineole, but the same compound could not be isolated from the urine of rabbits or rats. The hydroxy-cineole isolated was a mixture of several isomers which could not be separated, so the exact composition could not be determined. In properties and melting point two of the isomers resembled the cis and trans forms of 2-hydroxy-cineole.

Other products of detoxication could not be obtained.

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TABLE I

THE COMPARATIVE TOXICITY OF CINEOLE

	Number of Animals	Grams cineole per Kilo			L.D.50 per $\frac{a}{3}$ Kilo
		M.L.D.	L.D.50	Largest non Fatal Dose	
Guinea pig	31	0.7	1.35	2.6	1.2
Rabbit	31	1.2	1.9	3.2	2.3
Rat	31	1.6	3.2	4.6	2.5
Opossum	30	1.2	2.2	4.0	2.5

TABLE II

THE TOXICITY OF SINGLE DOSES OF CINEOLE IN OPOSSUMS

Dose: gm./kilo	0.9	1.2	1.5	1.8	2.1	2.4	2.7	3.0
No. Dead	0/8	1/8	4/8	2/8	2/8	3/8	3/8	5/8

TABLE III.

THE EFFECT OF CINEOLE ON THE EXCRETION OF GLUCURONIC ACID AND REDUCING BODIES

	Glucuronic Acid (mg./day)	Reducing Bodies (mg.glucose per day)	p.c. Recovery Cineole as Glucuronate	Cineole (gm.)
Rabbit	265	450	---	---
"	1430	630	44	2.9
Guinea Pig	35	60	---	---
" "	90	90	14	0.4
Opossum	70	175	---	---
	1020	440	44	1.8

TABLE IV.

RECOVERY OF CINEOLE AS GLUCURONATE IN AN OPOSSUM

Cineole (gm.)	mg. Glucuronate	% Cineole accounted for
0.3	140	36
0.8	500	53
1.3	750	46
1.8	1020	44
2.0	1280	48
3.2	3200	80

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THE EFFECT OF SALICYLATES (A) ON THE ESTIMATION OF THIAMINE BY THE THIOCHROME METHOD, (B) ON THE EXCRETION OF THIAMINE

by JOAN B. CLELAND

THE EFFECT OF SALICYLATES (A) ON THE ESTIMATION OF THIAMINE BY THE THIOCHROME METHOD, (B) ON THE EXCRETION OF THIAMINE

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A. THE EFFECT OF SALICYLATES ON THE ESTIMATION OF THIAMINE BY THE THIOCHROME METHOD.

Using the thiochrome method of Wang and Harris (1939) for estimating the thiamine content of 24-hour specimens of urine from hospital patients, it was found that when salicylates were present the isobutanol extracts gave a blue fluorescence which resembled and masked that given by thiochrome itself; in some cases the blank showed greater fluorescence than the unknown. The taking of a single aspirin tablet during the test was sufficient to make the titrations difficult or impossible to read accurately. This interference by salicylates has not been recorded in the literature. If, however, the urine was made markedly acid with concentrated HCl almost all the salicylates could be removed by a preliminary extraction with isobutanol. The titrations could then be carried out satisfactorily and the Gerhardt test was found to be negative or faintly positive. When larger quantities of salicylates were present (giving a purple colour with ferric chloride) it was sometimes necessary to extract the acidified urine twice or even three times with isobutanol. Urines which gave a marked positive test with ferric chloride in dilutions of 1 in 10 required still more extractions, and recovery of added thiamine was poor. As Wang and Harris (1939) recommend a preliminary extraction of the urine with isobutanol to remove other fluorescent compounds, this method of removing salicylates takes little extra time, and is satisfactory for all except urines containing very large amounts of salicylates.

The recovery of added thiamine from urine containing salicylates and treated with acid was much greater than from the unacidified urine (Table 1), and except with very large amounts of salicylates was almost as great as that from normal urine or normal acidified urine (Table 2).

Method.

Four estimations were carried out on each specimen of urine using the following dilutions: (1) 6 c.c. urine + 2 c.c. water. (2) 6 c.c. urine + 1 c.c. water + 1 c.c. concentrated HCl. (3) 6 c.c. urine + 1 c.c. water + 1 c.c. pure thiamine solution (containing 1 or 2 γ thiamine). (4) 6 c.c. water + 1 c.c. concentrated HCl + 1 c.c. pure thiamine solution. Each urine was then given one or more preliminary extractions with 5 c.c. of isobutanol and the thiamine determined by the method of Wang and Harris (1939).

Results.

TABLE 1.

Average of thiamine estimations on 22 urines containing salicylates.

	Before addition of acid.	After addition of acid.
Average thiamine in 8 c.c. urine (γ)	0·2 (0 to 1·6)	0·54 (0 to 1·5)
Average thiamine in 8 c.c. urine after addition of 2 γ thiamine (γ)	0·78 (0 to 2)	1·76 (1·3 to 2·6)
Average p.c. recovery	30 (0 to 60)	62 (43 to 90)
Gerhardt test	+ to +++	— to ±

TABLE 2.

Average recovery of thiamine from normal urine.

No. of samples.	On normal urine. Thiamine added per sample (γ).	Average p.c. recovery.	After addition of acid. Thiamine added per sample (γ).	Average p.c. recovery.
7	1	77	1	81
4	2	63	2	63

Better recovery of added thiamine has been obtained by using relatively larger quantities of methanol. Harris and Wang (1941) found that only 50–70 p.c. of the true value for thiamine was obtained when methanol comprised less than 50 p.c. total volume before the addition of the sodium hydroxide. In the experiments already described the methanol amounted to only 33–44 p.c. of this volume. When the volume of methanol was increased the results shown in Table 3 were obtained.

TABLE 3.

Influence of volume of methanol on the recovery of added thiamine from urine.

	No. of samples.	Thiamine (γ) added per sample.	Salicylates (before extraction).	Average p.c. recovery of added thiamine. Methanol > 50 c.c. volume.	Methanol < 50 p.c. volume.
Without acid	3	2	—	90	69
	3	1	—	94	80
	7	0·4	—	99	89
	3	0·4	++ to ++++	0*	0*
	3	1	++ to ++++	0	0
	3	2	++ to ++++	0*	0*
After acid	3	0·4	++++	99	91
	4	1	++++	90	61
	5	2	++++	94	75

* In one case blank tube showed greater fluorescence than unknown.

B. THE EFFECT OF SALICYLATES ON THE URINARY EXCRETION OF THIAMINE.

Samuels *et al.* (1940) reported an increased excretion of ascorbic acid when salicylates were given to guinea-pigs and rats. Daniels and Everson (1936) and Keith and Hickman (1938) noted a similar increase in excretion with children though Youmans (1937) failed to confirm this effect in adults. It has been known for some time that salicylates increase the urinary excretion of total nitrogenous substances and uric acid (Grabfield and Knapp, 1928) and allantoin (Grey and Grabfield, 1934). This section reports the effect of salicylates on the excretion of thiamine.

During the routine examination of the thiamine excreted in 24-hour samples of urine from a number of hospital patients, it was noticed that in five patients low, or relatively low, values were recorded during prolonged therapy with sodium salicylate. On the other hand in four out of six cases relatively high values were obtained after single doses of, or short periods of treatment with, salicylates.

Method.

The thiamine was estimated by the method of Wang and Harris (1939) after the salicylate had been removed by the method already described.

Results.

In Table 4 the thiamine excreted by five cases of rheumatic fever in whom treatment with sodium salicylate had been continued for ten or more days is shown. In all cases low or relatively low values were obtained.

In Fig. 1 the thiamine excreted per day and the effect on thiamine excretion of single doses of salicylates are recorded in five other cases. In three of these an increased excretion after salicylates was noted. The clinical history of cases 7 and 11 was not significant, but case 8 had been taking up to seven A.P.C. tablets a day for several weeks. The last A.P.C. tablet was taken twenty-four hours before the test was started. Both the cases which showed no response had

TABLE 4.

Effect of salicylate therapy on urinary excretion of thiamine.

Case No.	Before salicylate therapy. Average daily excretion of thiamine (γ).	During salicylate therapy. Daily excretion of thiamine (γ).	Number of days since salicylate therapy started.	Na salicylate taken each day (grains).	After salicylate therapy. Daily excretion of thiamine (γ).	Number of days since salicylate last taken.
1	181*	0 and 0	17 and 21	50	—	—
2	124*	0 and 0	11 and 12	50–60	95 and 96	10 and 11
3	129†	105 and 70	32 and 33	90	—	
4	230†	88 and 112	40 and 41	50	—	
5	100*	—	—	30	88 and 86 138	3 and 4 8

* Average from patients in same ward. † Average from same patient before treatment.

recently shown symptoms possibly due to deficiency, the one of thiamine itself and the other of vitamin B complex. The case notes of these two subjects are:

Case 9. Man, aged 32, who for two months had been having a poor diet and intermittent numbness of the 4th and 5th fingers of the left hand. The symptoms disappeared after receiving 4 mg. thiamine hydrochloride t.i.d. for 2 weeks, but reappeared 3 weeks later (about 6 weeks before this estimation of thiamine in the urine).

Case 10. Woman, aged 51, with achlorhydria and migraine. For several years has had recurrent attacks of fibrositis or "neuritis" and cheilosis. Usually has a high carbohydrate diet with fruit and vegetables but little meat and few eggs. Has occasionally taken supplements of vitamin B complex (Combex) with general improvement in health.

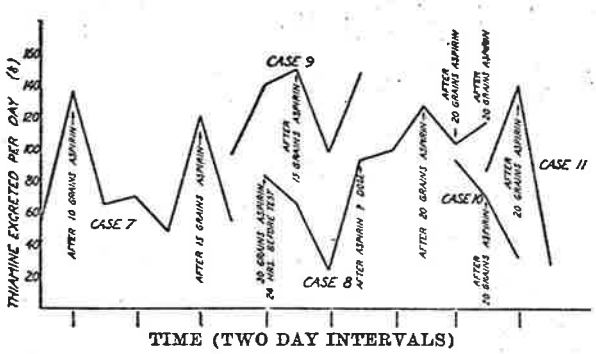

Fig. 1. Effect of single doses of salicylates on excretion of thiamine. (In each case aspirin was taken during the first twelve hours of the twenty-four hour collection period.) For particulars of cases see text.

Another case (Case 6 not shown in Fig. 1) received 50 grains aspirin twenty-four hours before the test was started, but none during the test. The average thiamine excreted per day for the next two days was 94 micrograms. He then received 50 grains sodium salicylate per day for five days. During the 5th day 180 micrograms thiamine were excreted.

Experiments were then carried out to see whether the low values obtained after salicylate therapy, or the high values obtained after single doses could be attributed solely to the salicylates.

The thiamine excreted in the urine each day by 9 subjects (five normal persons and four hospital patients with no evidence or likelihood of thiamine deficiency) was measured over two periods of five consecutive days. At the beginning of the third day of each period the subject received an intramuscular injection of either 1 or 2 mg. thiamine. The second five-day period was started on the same day of the week as the first period (i.e. two days after the completion of the first period) so that by arrangement the menu offered on corresponding days of each period was practically the same. During one of the five-day periods the subject received 10 to 30 grains of aspirin or sodium salicylate each day, the first dose being taken at the beginning of the test.

TABLE 5.

Thiamine excretion per day for five days with and without taking salicylates.

Subject.	Salicylates taken during first 5-day period.						Salicylates taken during second 5-day period.											
	I		II		III		IV		V		VI		VII		VIII		IX	
Particulars.	M. age 51, R. hemiplegia.		F. age 26, normal.		F. age 25, normal.		F. age 12, Psoriasis.		F. age 37, T.B. abscess.		F. age 17, T.B. lesion of skin.		M. age 20, normal.		F. age 29, normal.		F. age 62, normal.	
Salicylate (s) / Aspirin (a) grains taken per day.	45(s) —		20(s) —		10(a) —		—	30(s)	—	30(s)	—	30(s)	—	20(a)	—	10(s)	—	15(s)
Thiamine excreted per day (micrograms) 1st day	174	115	141	104	141	105	160	106	56	158	65	92	199	280	114	171	—	—
2nd day	150	98	136	45	154	90	85	115	92	170	183	100	81	141	119	102	194	170
3rd day*	630	236	231	120	221	182	154	180	340	350	138	540	300	480	115	441	330	414
4th day	150	118	216	93	178	137	72	163	186	190	256	154	196	265	138	113	220	288
5th day	112	108	201	98	128	90	74	241	150	306	42	84	146	238	200	113	156	342
*Thiamine injected on 3rd day (mg.)	2	2	1	1	1	1	2	2	2	2	1	1	2	2	2	2	2	2
Ratio of thiamine excreted in 5 days with salicylate: Thiamine excreted in 5 days without salicylate. as p.c.	180		201		136		146		143		142		152		137		135	

In Table 5 the thiamine excreted each day in each of the nine test cases is recorded. These results have been examined statistically; in each case there was a significant rise in the thiamine excreted while salicylates were being taken. (Mean daily excretion of thiamine without salicylates 144 γ; with salicylates 216 γ. Standard error $\pm$ 10.) This increase was of about the same order as the increased excretion of uric acid and total nitrogen (Grabfield and Knapp, 1928), and of ascorbic acid (Daniels and Everson, 1936; Keith and Hickman, 1938) found under similar circumstances; but it could not be correlated with variations in the volume of urine excreted.

As approximately the same results were obtained with or without taking into account the increased excretion due to the injection, the salicylates must affect the excretion rather than the absorption of the vitamin. In two cases (subjects 6 and 8), however, the total increased excretion of thiamine with salicylates could be accounted for by the increase occurring in the 24 hours following the injection of thiamine; but in both these subjects there was little or no immediate response to the injection under normal conditions.

DISCUSSION.

Although the number of cases is insufficient to warrant wide generalizations, the results shown in Table 5 and Fig. 1 indicate that salicylates may have a marked effect in increasing the urinary excretion of thiamine over short periods. This increased excretion is not the result of increased absorption.

In some cases (Case 5, Table 4, Case 6; Case 8, Fig. 1) the withdrawal of salicylates was followed by a reduced thiamine output for a few days, presumably until the depleted body stores were replenished. The large differences between the values obtained with and without salicylate treatment in subjects 1 and 2 (Table 5) are also probably due to this effect, as in both these cases the period with salicylates preceded the normal period. The only two cases in which no appreciable rise in thiamine excretion occurred immediately after taking salicylates (Cases 9 and 10, Fig. 1) had both recently shown symptoms attributable to vitamin B deficiency; it seems reasonable to suggest that in these cases the tissue stores had already been depleted or were incapable of being mobilized.

Samuels, Ritz *et al.* (1940) found in rats, following the withdrawal of salicylates, a similar fall in the excretion of ascorbic acid below the original control level. They also observed that guinea-pigs receiving less than a certain quantity of ascorbic acid in their food failed to excrete larger quantities of ascorbic acid when salicylates were given.

The amount of thiamine excreted in the urine in 24 hours depends therefore not only on the thiamine status of the individual at the time, but also on whether salicylates have been taken just prior to or during the test. Variations in thiamine excretion due to salicylate administration will not alter the clinical interpretation of the results if the thiamine status of the patient is very low or very high, but with borderline cases it may have a marked effect. Many people take aspirin or A.P.C. tablets frequently and, in particular, for conditions such as "neuritis" where an estimation of the thiamine status might be of use to clinicians. It is possible that some of the inconsistencies observed in this method of evaluating the status may be due to the effect of salicylates. More constant agreement has been obtained between the quantities of thiamine excreted in two consecutive 24-hour specimens of urine since patients have been asked to refrain from taking salicylates for several days prior to and during the collection of the samples. (When necessary some other analgesic was supplied.)

Longer periods of treatment with large doses of salicylates (as in rheumatic fever) appear to lead to a reduced thiamine output, presumably due to a loss of body stores during the preliminary period of increased excretion (see Table 4). It is, however, a little difficult to reconcile these low figures with the absence of any symptoms which could be attributed to thiamine deficiency. No records of thiamine deficiency associated with rheumatic fever have been found, though Rinehart (1935) has recorded a number of cases of scurvy associated with rheumatoid arthritis treated with salicylates. Possibly the symptoms of salicylate poisoning develop more rapidly than do those of thiamine deficiency, so that salicylate treatment is stopped before the latter are recognized. It is also possible that the low thiamine excretion is due to a raised renal threshold, and that the mobilization of the body stores of thiamine is partially responsible for the action of salicylates in

relieving pain. It seems reasonable to suggest that care should be taken to ensure that patients undergoing salicylate therapy should have an extra supplement of thiamine.

SUMMARY.

Salicylates give a blue fluorescence in ultraviolet light and therefore interfere with the thiochrome method of estimating thiamine. They are not removed by the usual preliminary extraction with isobutanol unless the solution has been made markedly acid.

Over short periods salicylates increase the excretion of thiamine in urine, but long periods of therapy cause a reduced output. The significance of these results on the interpretation of the thiamine excretion test is discussed. It is suggested that a high thiamine intake is advisable during salicylate therapy.

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THE EFFECT OF SALICYLATES AND THIAMINE DEFICIENCY ON THE THIAMINE CONTENT OF RAT TISSUE

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In a previous experiment (Cleland, 1943), it was found that in the few cases studied, the administration of salicylates was associated with an increase in the amount of thiamine excreted in the urine of humans. Under similar conditions an increase in the excretion of ascorbic acid was noted by Daniels and Everson (1936) and Keith and Hickman (1938), while Samuels *et al.* (1940), found that in rats the increased excretion of vitamin C was associated with a loss of it from the liver and brain. The present paper reports the effect of the administration of salicylates on the thiamine stores of rats.

METHOD.

Experiments were made using both young and adult rats which were fed on either the normal stock diet or a thiamine deficient diet of the following composition:

20	parts	casein
70	„	sucrose
5	„	salt mixture (Harris, 1938)
10	„	dried autoclaved yeast (Evans *et al.*, 1929)
3	„	cod liver oil substitute

Adult rats of the same sex were divided into pairs of approximately the same age and weight. One rat of each pair received by stomach tube 0·5 c.c. 10 p.c. sodium salicylate each day for eighteen days, while the other received 0·5 c.c. distilled water at the same time and under the same conditions The animals were killed after eighteen days and the thiamine content of various tissues was determined.

When young rats (2 months old) were used, the amount of salicylate given had to be reduced to 0·3 c.c. 5 p.c. sodium salicylate given four times a week. This was administered by means of an adapted syringe needle made to the directions given by Ferril (1943). These young rats were fed on either the deficient or the normal stock diet and, as with the adult rats, those on each type of diet were divided into pairs of approximately the same age and weight, one of which received sodium salicylate, the other an equivalent amount of water. Those on the normal diet were killed after receiving treatment for six weeks, while those on the deficient diet were left until they died (4–8 weeks), and the tissues were then removed for analysis.

Brain, liver and kidney were the tissues chosen for analysis because the thiamine content of the two latter has been shown to vary rapidly with alterations in thiamine intake (Ferrebee *et al.*, 1942), and would therefore be more likely to reflect variations in the thiamine status of the rat, while Samuels *et al.* (1940), found that when rats received salicylates there was a loss of ascorbic acid from the liver and the brain.

The fresh tissues were prepared for analysis by the method described by Ferrebee *et al.* (1942), for human tissue, except that smaller amounts of tissue and reagents were used and the tissue was finely ground with acid washed sand instead of using the Waring blendor. Alexander (1943), however, has found that grinding the tissues extracted as much thiamine as did dispersion in the blendor. The determination of thiamine in the final extract was done by the thiochrome method of Wang and Harris (1939).

RESULTS.

The average and range of values obtained for liver, kidney and body weight and for thiamine concentration in liver, kidney and brain for each different group of rats are shown in Table 1. These have been examined statistically.

TABLE 1.

Weight and thiamine content of rat tissues.

No. of rats.	Age.	Received.	Diet.	Weight (gm.). Total.	Liver.	Kidney.	Thiamine concentration (γ/gm.). Liver.	Kidney.	Brain.	
12	adult	water	normal	203	9·04	1·55	3·00	1·78	0·91	Average
				154–276	6·81–11·65	1·40–2·0	1·96–4·83	0·81–3·14	0·58–1·41	Range
12	adult	salicylates	normal	198	9·54	1·56	3·91	2·06	0·97	Average
				148–262	7·76–13·04	1·42–2·24	2·40–7·71	1·26–3·18	0·45–1·48	Range
12	adult	water	thiamine deficient	195	9·35	1·86	0·32	0·39	0·56	Average
				156–255	7·17–13·10	1·57–2·17	0·20–0·43	0·22–0·49	0·39–0·77	Range
12	adult	salicylates	thiamine deficient	198	11·63	2·07	0·40	0·39	0·53	Average
				156–255	8·18–14·79	1·75–2·54	0·29–0·81	0·22–0·73	0·40–0·70	Range
12	young	water	normal	139	8·09	1·12	2·16	2·20	0·83	Average
				124–180	6·15–11·49	0·89–1·48	1·33–3·85	0·69–3·87	0·42–1·18	Range
12	young	salicylates	normal	144	8·47	1·25	2·18	1·94	1·03	Average
				116–171	5·89–11·44	0·92–1·52	1·69–2·79	0·84–2·96	0·45–1·59	Range
12	young	water	thiamine deficient	56	2·49	0·93	0·21	0·17	0·21	Average
				38–80	1·72– 3·23	0·63–1·44	0·12–0·40	0·09–0·33	0·10–0·35	Range
12	young	salicylates	thiamine deficient	63	2·74	1·07	0·15	0·21	0·21	Average
				41–80	2·01– 3·81	0·72–1·27	0·05–0·24	0·11–0·32	0·14–0·36	Range

With adult rats on a normal diet it can be seen that there was little difference between rats receiving salicylates and those receiving water in body weight, kidney weight or thiamine concentration in the brain. The rats receiving salicylates, however, had a significantly greater (P less than 0·01) liver weight (the average increase being about 0·5 gm.) and thiamine concentration in the liver (average increase about 0·9 γ/gm.). The apparently increased concentration of thiamine in the kidney of 0·3 γ/gm. was not statistically significant.

Similarly, in the adult deficient rats there was an even greater increase in liver weight (average 2 gm.) and a smaller, but still significant rise in thiamine concentration in the liver of those animals receiving salicylates, while the other factors studied—body and kidney weight, and thiamine concentration in kidney and brain—were unaltered.

In young rats, however, on both normal and deficient diets, animals receiving salicylates did not differ significantly in any of these particulars, though they tended to be a little heavier. This was accompanied by an equivalent increase in organ weight so that the ratio of organ to body weight remained the same.

In the young rats the rate of development of thiamine deficiency was not affected by the administration of salicylates. It was also found that on the normal stock diet the older rats had a significantly greater (P less than 0·01) concentration of thiamine in the liver than the young rats, the average value obtained from all the adult rats being about 3·5 γ/gm. and from all the young rats about 2·2 γ/gm. In both age groups the concentration was about the same in the kidney (ca. 2·0 γ/gm.) and the brain (ca. 0·9 γ/gm.).

On the other hand, when animals were fed on the thiamine deficient diet the thiamine concentration in the brain of adult rats (ca. 0·5 γ/gm.) exceeded the concentration in other tissues and was significantly greater than the concentration in the brain of the young deficient rats. In the adult rats the concentration in both liver and kidney was about 0·4 γ/gm. while in the young deficient rats the concentration in all three tissues was about 0·2 γ/gm.

As would be expected, in the young deficient rats there was a greater ratio of both liver and kidney weight per gm. body weight than in the control group on the normal diet.

DISCUSSION.

The most significant finding of these experiments is the relatively greater weight of the liver of those adult animals which received salicylates. Macroscopically the livers of these animals were not engorged and sections of three of them showed no histological abnormality and did not differ materially from those of the corresponding control animals. Because the thiamine concentration in these

organs was increased it seems most likely that the greater weight was due to hypertrophy rather than to deposits of fat, water or glycogen which, presumably, would not be associated with the retention of thiamine. It is curious, though, that no records of the effect of salicylates on organ weight can be found in the literature. Samuels *et al.* (1940), in similar experiments on ascorbic acid excretion in the rat, record the organ weight (but not body weight) only of animals which had received salicylates for 1–3 days. It is unlikely that this would be sufficient to provoke hypertrophy, and the results given by them show no significant difference in this respect between treated and untreated animals.

In humans, Kapp and Coburn (1942) found that 80 p.c. of ingested salicylate could be recovered in the urine with the salicyl radicle intact. Of this 55 p.c. was conjugated with glycine and 25 p.c. with glucuronic acid. Assuming that in the rat the liver is the chief site of detoxication, the relative increase in the weight of liver could be attributed to the increased strain thrown on it by the need for salicylate detoxication. Lipschitz and Bueding (1939), have shown that rat liver slices are capable of producing conjugated glucuronates and that production was greatest when pyruvic acid or dihydroxyacetone was used as substrate. As thiamine is necessary for normal utilization of pyruvic acid it is probably required for glucuronate synthesis. Martin *et al.* (1943), found that in rats thiamine deficiency reduced glucuronic acid excretion. Lutwak-Mann (1942), however, failed to find an increased excretion of glucuronates when salicylates were injected into rats, nor did she find that glucuronates were produced *in vitro* by rat liver slices in the presence of salicylate though they were formed when p–hydroxybenzoic acid was used. On the other hand she found that *in vitro* M/10 salicylate affected the carboxylase system and the dismutation between hexosediphosphate and pyruvate. The fact that injections of salicylate were associated with a sudden rise in liver glycogen levels indicates that it is somehow connected with carbohydrate metabolism (Lutwak-Mann, 1942).

The increased concentration of thiamine in rat liver does not suggest an explanation for the increased thiamine excretion found when salicylates were given to several normal people. In fact, the administration of salicylates appeared to increase the efficiency with which thiamine was stored (to a limited extent) in adult rats. This, however, did not occur in young rats, and symptoms of thiamine deficiency occurred just as readily in young deficient rats receiving salicylates as in the control animals which were given water.

The thiamine concentration of the tissues of young deficient rats probably indicates the minimum concentration compatible with life, since these figures were obtained from animals which died of thiamine deficiency. It is of interest to note that under these conditions the concentration in the three tissues studied was about the same—that is, about 0·2γ per gm. In the adult rats in which the deficiency was not nearly so severe, the thiamine concentration in the liver and kidney was not significantly greater than in the young deficient rats, but the concentration in the brain was much higher. This suggests that the brain has first preference on available thiamine and that the content of the brain is less labile than that of other tissues. With a normal diet the concentration of thiamine in the liver was greater than in the kidney which was greater than in the brain, but when the diet was not adequate the greatest loss occurred from the liver and the least from the brain. These results are similar to those obtained by other workers (Ferrebee *et al.*, 1942; Schultz *et al.*, 1939; Sure and Ford, 1942; Sarett and Perlzweig, 1943; Schweigert *et al.*, 1943).

SUMMARY.

Administration of salicylates increased the liver weight per gm. body weight and the thiamine concentration in the liver of adult (but not young) rats, on both normal and thiamine deficient diets. Thiamine concentration in kidney and brain, kidney weight, and the rate of development of symptoms of thiamine deficiency in young rats were not altered by giving salicylates.

In young thiamine-deficient rats the thiamine concentration in brain was lower than in adult deficient rats but the concentration in kidney and liver was

much the same; on a normal diet the concentrations in kidney and brain were similar in adult and young rats, but the thiamine concentration in the liver was significantly higher in adult rats.

Possible interpretations of these results are discussed.

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