A PRELIMINARY REPORT ON THE METABOLISM OF S-AMINOETHYLCYSTEINE BY THE RAT *IN VIVO**

by

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INTRODUCTION

S-Aminoethylcysteine (AEC) was synthesized at about the same time by CAVALLINI, DE MARCO, MONDOVÌ AND AZZONE[1] and by ELDJARN[2] with the purpose of testing its role in the biological transulphuration from cysteine to ethanolamine. The mechanism by which mercaptoethylamine (cysteinamine), necessary for the biological synthesis of the CoA, is secured by the organism is still unknown; recent work of THOMPSON AND BIRD[3] would exclude that cysteine, at least in the form of a peptide linkage with pantothenic acid, is used by the rat. ELDJARN reported the sulphur partition and the content of taurine in the urine of rats after injection of AEC, but at yet no evidence is given on the cleavage of AEC to cysteinamine or cystamine. In the present paper we report our first results in the study of the metabolism of AEC, *in vivo* by the rat, showing that the injection of AEC is followed by the excretion in the urine of an appreciable amount of cystamine in a conjugated form. Furthermore a certain number of derivatives of AEC have been found, and tentatively identified.

MATERIALS AND METHODS

The animals used were male rats of the mixed breed of the laboratory. Throughout the experimental period they were kept in pairs in metabolic cages on the diet reported elsewhere[4]. The urine of any two animals was collected over 2 ml 2 *N* HCl in 24 h periods and diluted to 50 ml with the washings of the funnel and the bottom of the cage. AEC was injected, in the amount of 255 mg dissolved in 2 ml water, subcutaneously.

Unless otherwise stated, the conventional two-dimensional paper chromatography on Whatman No. 4 paper, using phenol and collidine-lutidine as solvents was used to detect ninhydrin-reacting compounds in the urine. The samples analysed were desalted by the Dent modification of the GORDON *et al.* electrolytic desalter[5]. The iodoplatinate reagent was used to detect sulphur-containing compounds[6]. The detection of organic disulphide compounds by filter paper chromatography was performed by spraying on the dried paper a freshly made mixture of the FOLIN-MARENZI reagent[7,8] and 10% $NaHSO_3$ (2/8 vol) and alkalinizing the paper over fumes of conc. ammonia. Column chromatography was the method standardized by STEIN[9] for human urine with the variant that the pH 2.5 buffer was substituted with 0.1 *M* citric acid containing 0.2 *M* NaCl[4,10,11], and thiodiglycol was omitted. The unknown compounds found by column chromatography were compared with the unknown found by paper chromatography by using the procedure of DRÈZE AND DE BOECK[12]. The FOLIN-MARENZI method as modified by SHINOHARA[13] was used for the quantitative determination of total disulphide-sulphur in the urine. The SULLIVAN AND HESS method was employed for the quantitative determination of the cystine disulphide-sulphur in the same material[14]. Other technical details will be given below.

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The synthesis of aminoethylcysteine has been reported elsewhere[1]. S-aminoethyl-(α, N-acetyl)-cysteine (AEAC) was prepared by a method similar to that used for AEC, starting from N-acetylcysteine. Acetylcysteine was obtained by reduction[15] of acetylcystine prepared as suggested by HOLLANDER AND DU VIGNEAUD[16]. The crude AEAC was purified by passing on to a column of Dowex-2. and eluting with 1 *N* acetic acid. The compound was recrystallized from hot absolute alcohol. M.p. 196–198°; S 15.54%, N 13.58%; found S 14.61%, N 13.25%. Cystamine was synthesized according to BARNETT[17]. Lanthionamine (the name given in this paper to the product of decarboxylation of lanthionine, in analogy to cystamine) and lanthionamine sulphone were synthesized according to COBLENTZ AND GABRIEL[18].

EXPERIMENTAL

Disulphide excretion after the injection of AEC

Four rats kept on the basal diet were injected with 255 mg AEC. The urines collected before and after the injection were analysed on 2 ml sample in duplicate for total disulphide-sulphur by the FOLIN-MARENZI method and for cystine sulphur by the SULLIVAN AND HESS method. A negative nitroprusside test in the urine has excluded the presence of any free thio-group in appreciable amount.

TABLE I

TOTAL DISULPHIDE-SULPHUR AND CYSTINE-SULPHUR IN THE URINE OF RATS BEFORE AND AFTER THE INJECTION OF AEC

Values: in mg S per 24 h per rat.

	Total -S–S– sulphur	*Cystine -S–S– sulphur*	*Non-cystine -S–S– sulphur*
24 h before the injection of AEC	0.68	0.28	0.40
24 h after the injection of AEC	2.72	0.80	1.92
48 h after the injection of AEC	0.89	0.32	0.57

As Table I shows in the first day after the injection of AEC there appears in the urine 1.92 mg of disulphide-sulphur not accounted for as cystine. The same experiment performed after the injection of an equal molar amount of cysteine did not give any appreciable excretion of non-cysteine disulphide-sulphur. By subtracting from 1.92 mg of sulphur the 0.40 mg present in the urine of untreated rats one obtains a figure of 1.52 mg of disulphide-sulphur excreted in an unknown form which calculated as cystamine dihydrochloride would account for 6.7 mg of this compound *i.e.* for a cleavage of 4.7% of the AEC injected. This experiment was repeated with essentially the same results.

Paper chromatography

Paper chromatography on 0.4 ml sample of the diluted urines of AEC injected rats, compared with the urine of normal rats kept on the same diet, showed the presence of a number of unknown substances; Fig. 1a and b. The amino acids which are normal constituents of rat urine have been marked in the figures by numbers, the unknown substances by letters. Equal amounts of urine of both the injected and untreated rats were chromatographed after 15 h hydrolysis with ¼ vol conc. HCl and after removal the excess of acid by vacuum distillation; Fig. 1c and d. The hydrolysis of normal urines caused the appearance of phenylalanine and tyrosine and the increase of all the other

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spots. The hydrolysis of rats injected urines was followed by the appearance of the new spot E, the disappearance of spot A and the increase of spot B, as far as it regards the new compounds; the other spots behaved quite similarly to the spots of normal urines.

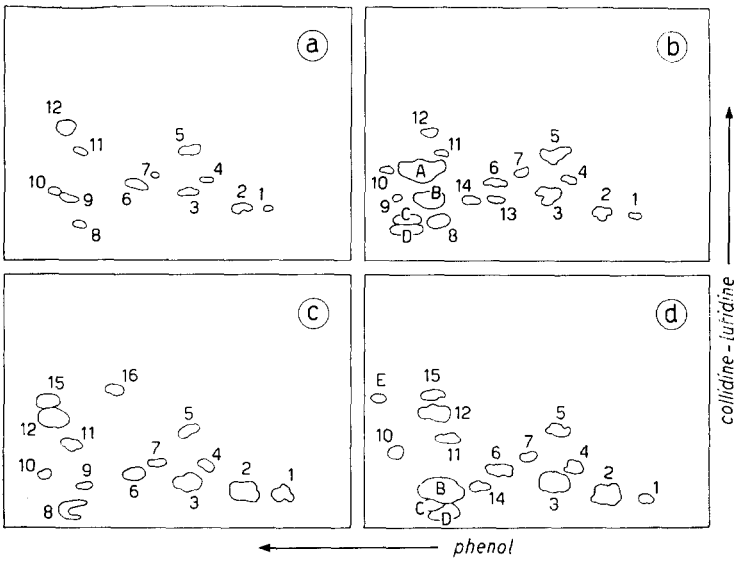

Fig. 1. Paper chromatography for the amino acids of the urine of rats injected with aminoethylcysteine. 0.4 ml of 24 h urine of 2 rats, diluted to 50 ml were dried in the bottom right corner. (a) non-injected rats; (b) injected rats; (c) urine of non-injected rats after 15 h hydrolysis; (d) urine of injected rats after 15 h hydrolysis. Key for amino acids: 1, Asp.; 2, Glu.; 3, Gly.; 4, Ser.; 5, Tau.; 6, α-Ala.; 7, Thr.; 8, Lys.; 9, γ-Am.but.; 10, Carn.; 11, Val.; 12, Leu.; 13, Glu.a.; 14, β-Ala.; 15, Phe.; 16, Tyr.; A, B, C, D, E, unusual ninhydrin-reacting, sulphur-containing compounds.

The sulphur-containing amino acids have been detected by developing the chromatograms with the iodoplatinate reagent. Positive reactions have been obtained with all the A, B, C, D, and E spots, showing that they must be derivatives of the injected AEC. The test for organic disulphide compounds by the FOLIN-MARENZI spraying solution was positive only with spot E. The migration coordinates of spot E and the positive test with the iodoplatinate and the FOLIN-MARENZI reagents strongly suggest that this spot might be identified as cystamine. That cystamine does not arise by the HCl hydrolysis of the AEC eventually present in the urine was shown by hydrolysing a synthetic sample of AEC under the same conditions used for the hydrolysis of urine and by chromatographing on paper the dried residue: AEC was shown to be stable to hydrolysis and no cystamine or cysteinamine were detected.

After the hydrolysis of urine spot A disappears. The only spot which increases in comparative amount is spot B, showing a relation between these two compounds. In order to establish unequivocally the derivation of compound B from A, as a result of hydrolysis, compound A has been isolated by paper chromatography, hydrolyzed, and rechromatographed as follows. Five ml of AEC-treated rat urines were dried on the top of a large sheet of Whatman No. 4 paper as a continuous band. The paper was run in phenol and dried. Two narrow lateral strips were cut out of the paper and developed with ninhydrin in order to check the position of compound A. From the not-developed residual paper, a band containing the compound A was cut out taking as

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reference the developed lateral strips. The band was eluted with water and the solution was dried again on the top of a sheet of Whatman paper. The new chromatography was performed with collidine-lutidine and the localization and the elution of compound A were done as in the case of phenol. The final solution, containing only the compound A as the main component, was used for the following tests. (1) A portion was chromatographed without any treatment and was used as reference: this gave a ninhydrin- and iodoplatinate-positive spot with the same migration characteristics as spot A. (2) A portion was hydrolyzed under reflux with ¼ vol conc. HCl for 15 h, dried to remove the HCl excess, and rechromatographed with and without the addition of a synthetic sample of AEC. After hydrolysis spot A gave a compound with the R_F of spot B, which was indistinguishable from the added synthetic AEC. No other ninhydrin reacting compounds appeared after hydrolysis, so that compound A has to be considered a conjugate form of AEC bound with a non-amino-containing compound, probably an acetyl derivative. In order to establish which one of the two aminogroups of AEC is involved in the linkage, another portion (3) of the extracted compound A was chromatographed on copper carbonate dusted paper, used for distinguishing α-from not α-amino acids[19]. With copper carbonate the compound travelled to the same position showed in the unpretreated paper. Since AEC is retained by copper carbonate we have argued that the α-NH_2 group of the cysteine moiety must be the conjugated group. Synthetic aminoethyl-(α,N-acetyl)-cysteine was prepared and it was shown to have the same R_F and the same properties as the compound A.

Of the new spots C and D only compound C resembles in position and properties lanthionamine. In Table II data are given of all the characteristics of the new compounds found by paper chromatography.

TABLE II

Spot	*Ninhydrin*	*Iodoplatinate*	FOLIN-MARENZI	*Hydrolysis*	*$CuCO_3$*	*R_F equal to*
A	+	+	—	D	M	aminoethyl-(α, N-acetyl)-cysteine
B	+	+	—	I	R	aminoethyl-cysteine
C	+	+	—	S	?	lanthionamine
D	+	+	—	S	?	?
E	+	+	+	A	M	cystamine

D = disappears; I = increases; S = stable; A = appears; M = moves; R = retained.

As a result of the paper chromatography experiments we may conclude that there is a strong evidence for the identification of spot A as AEAC, spot B as unchanged AEC, spot E as cystamine and spots C and D as sulphur-containing derivatives of AEC. Only spot C checks with lanthionamine and, though its position is ambiguous, may be tentatively identified as lanthionamine. We have no sound indication for the identification of spot D.

Column chromatography

Similar samples of urine have been analysed by column chromatography. Figs. 2 and 3 show the chromatograms of hydrolyzed and unhydrolyzed urine of rats injected with AEC and equal samples of the urine of rats used as control. The resolution of the neutral and acidic amino acids has been performed on a 0.9 × 100 cm column, the basic

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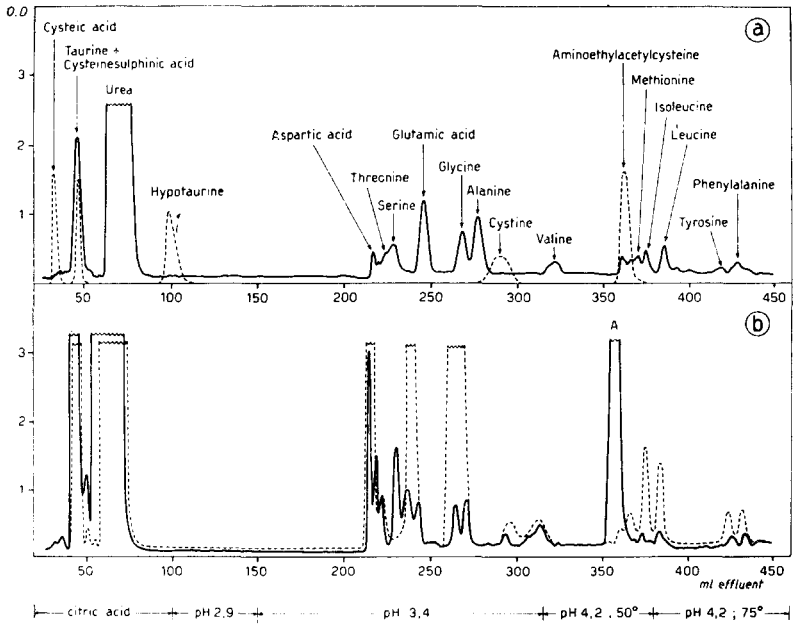

Fig. 2. Ion-exchange chromatography on 0.9 × 100 cm column on 4 ml of 24 h urine of two rats, diluted to 50 ml, following STEIN[9]. (a) Urine of normal rats. The position of some synthetic derivatives of cystine is indicated by dotted lines. (b) Urine of rats injected with aminoethylcysteine. The dotted line shows the chromatogram of the same urine after 15 h hydrolysis. Capital letters mark the same compounds found by paper chromatography.

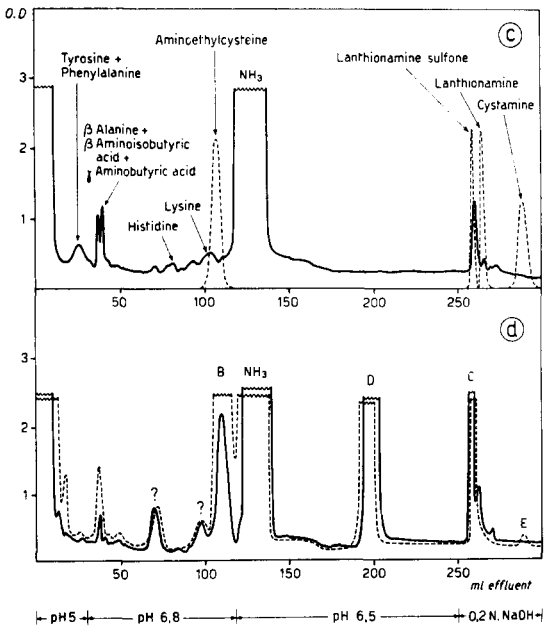

Fig. 3. Ion-exchange chromatography on 0.9 × 15 cm column of the same urines of Fig. 2. (c) Urine of normal rats. Dotted lines, synthetic compounds. (d) Urine of rats injected with aminoethylcysteine. Dotted line shows the same urine after 15 h hydrolysis. Conditions as in Fig. 2.

amino acids have been resolved on a 0.9 × 15 cm column. In the chromatograms of normal rat urines the position of some compounds which are pertinent to the present research is indicated by a dotted line. Besides the increased excretion of taurine, already reported by ELDJARN, the main difference found by column chromatography between the urine of injected and not-injected rats consists in the appearance of four large peaks in the regions of the leucines, ammonia, arginine and after the appearance of the NaOH front. Furthermore a small peak in the same region as synthetic cystamine appears after hydrolysis. The identification of the new peaks detected by column chromatography on the injected rats urine with the new spots found by paper chromatography has been achieved by re-

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chromatographing on paper the fractions collected under the peaks after having removed the components of the eluting buffer as suggested by DRÈZE AND DE BOECK[12]. In the diagrams the identity of the compounds is made apparent by using the same letters as in the paper chromatograms. In any case the isolated compound had the same R_F and the same reactions for sulphur as the corresponding one on paper. The hydrolysis caused also in column chromatography variations similar to that observed on paper, namely the disappearance of peak A, the increase of peak B and the appearance of the small peak E. The position in the column chromatograms of the new peaks found after injection of AEC corresponds satisfactorily to the position of the synthetic compounds to which the peaks have been tentatively identified, owing to the correspondence with the spots found on paper. This strongly supports the provisional identification reported in the last column of Table I. The remaining small peaks found in the column chromatograms of the AEC-injected rat urine have not been considered in the present work.

Extraction and provisional identification of cystamine

The results of the analysis of the disuphide-sulphur content in the urine of rats after injection of AEC and the results of the paper and column chromatography corroborate the conclusion that among other compounds cystamine is also excreted. Since cystamine appears in the chromatograms after hydrolysis of the urines, it must be excreted in a conjugated form. We have succeded in extracting from the urine small amounts of cystamine of different degrees of purity which were used to check its identity with the synthetic compound. The best results have been obtained using the following procedure.

The pooled urines of four rats injected with AEC, collected over 4 ml 2 *N* HCl during a period of 24 h after the injection, were hydrolyzed for 14 h with ¼ vol conc. HCl at reflux. The hydrolyzate was dried in the vacuum over a boiling bath, dissolved in a small amount of water, decolorized with charcoal and transferred to a column of Amberlite IR-100 in the H-form, 1 × 30 cm bed, 80–100 mesh. The column was washed with water until the effluent was neutral, then eluted with 500 ml 5 *N* HCl. The eluate was dried, dissolved in a small amount of water and transferred to a column of Dowex-2 in the OH-form, 1 × 20 cm bed, 80–100 mesh. The percolate and the following washing with 200 ml water were collected, acidified with conc. HCl and dried in vacuum over a boiling bath. Cystamine was reduced by dissolving the residue in water, adding 4 ml conc. HCl and *ca.* 2 g of zinc dust. After shaking for 30 min the residual zinc was filtered off and 10 ml 4% $HgCl_2$ were added to the solution. After standing overnight at room temperature a crystalline precipitate was collected on a filter, washed with 100 ml water suspended in 15 ml water, and decomposed by bubbling in H_2S for 1 hour. The precipitated HgS was removed by filtration, washed with H_2S saturated water and the solution, to which a few drops of conc. HCl had been added, was dried. The residue was taken up in 5 ml water and oxidized with iodine. To avoid any excess of iodine the oxidation was carried out with iodine dissolved in chloroform and the solution was shaken until a further addition of chloroform was not discoloured. The water layer was removed, extracted with ether, a few drops of conc. HCl were added and the solution was then dried. In order to remove any excess of HI, the residue was taken in conc. HCl and dried many times. Roughly 10 mg of a white powder were obtained giving a strong nitroprusside test after treatment with NaCN. The above procedure applied to the urines of non-injected rats gave a negative result.

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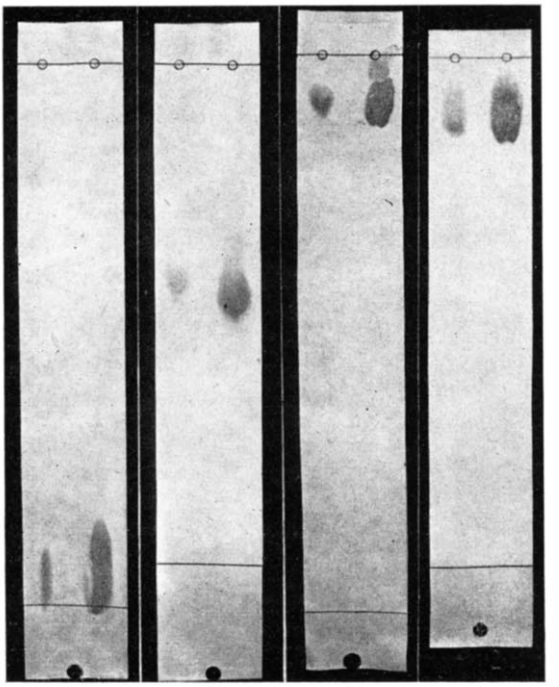

Fig. 4. Paper chromatography of cystamine extracted from the hydrolized urine of rats injected with aminoethylcysteine compared with synthetic cystamine. For each chromatogram: at the left 50 μg of synthetic cystamine, at the right 280 μg of extracted cystamine. Solvents: 1, phenol; 2, collidine-lutidine; 3, butanol-acetic acid 20%; 4, butanol-ethanol 20%. Developing solution: FOLIN-MARENZI reagent.

The extracted cystamine was analysed by paper chromatography and by paper electrophoresis side by side with a synthetic sample of cystamine. In order to exclude the interference of impurities, after the chromatographic and the electrophoretic runs the paper strips were developed with the FOLIN-MARENZI reagent which is specific for the -S-S- bearing compounds. The chromatograms showing the identical behaviour of the synthetic and the extracted cystamine, with 4 different solvents, are given in Fig. 4. A typical result of 3 h paper electrophoresis on Whatman paper No. 1 with a Label apparatus in acetate buffer is shown in Fig. 5. Of particular significance is the identical behaviour of both the natural and the synthetic cystamine during electrophoresis. Of a number of thio- and disulphide-compounds tested, only cystamine and cysteinamine, being charged as cations, move towards the cathode in a wide range of pH at a relatively high speed.

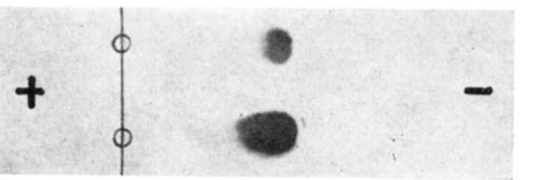

Fig. 5. Paper electrophoresis of 280 μg of cystamine extracted from the hydrolized urines of rats injected with aminoethylcysteine compared with 50 μg of a synthetic sample of cystamine. Above, the synthetic sample, below, the natural cystamine. The vertical line is the starting point. Acetate buffer pH 3.8, μ 0.05, V 6.08/cm, time 3 h. Developing solution: FOLIN-MARENZI reagent.

DISCUSSION

As a result of the experiments reported above we may conclude that AEC is metabolized by the intact rat and that only a small portion is excreted unchanged in the urine. Among the metabolic products the following sulphur-containing derivatives have been detected and tentatively identified: aminoethyl-(α, N-acetyl)-cysteine, cystamine in a conjugated form, a strong basic derivative probably lanthionamine, an unknown basic derivative. Furthermore the increased excretion of taurine has been confirmed. The scheme below shows the relations between the injected AEC and the compounds detected in the urine.

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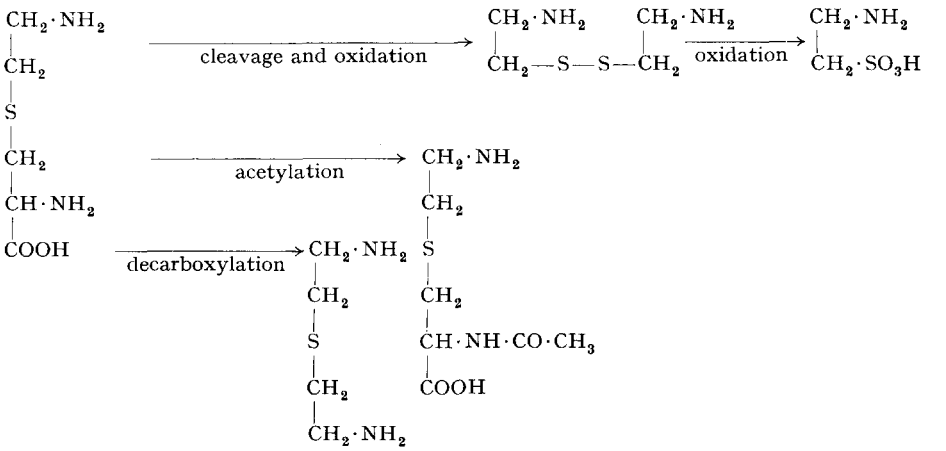

From the quantitative point of view, AEC acetylated in the NH_2 group of the cysteine moiety is the predominating product. Whether AEAC represents a detoxication compound or whether it has to be taken as a biological intermediate between AEC and other products, is a question which requires further investigation. The detection of cystamine in the urine after injection of AEC seems unequivocal and supports the hypothesis that AEC may be cleaved by the rat to yield mercaptoethylamine. The occurrence of a similar biological reaction with a number of S-derivatives of cysteine has already been reported by BINKLEY[20] and strongly corroborate this hypothesis. As far as it concerns the occurrence of a transulphurative reaction between cysteine and ethanolamine, with a model similar to the well known reaction between homocysteine and serine, the present paper may be taken as indicative in showing the biological feasability of the second part of the transulphurative process. The demonstration of the occurrence of all the complete reaction will be experimentally supported by showing the synthesis of AEC or of its acetylated derivative as a result of the interaction of cysteine and ethanolamine in a biological medium.

SUMMARY

Synthetic S-aminoethylcysteine has been injected into white rats and the urines have been analyzed for total disulphide-sulphur by the FOLIN-MARENZI method and for cystine-sulphur by the SULLIVAN AND HESS method. An amount of non-cystine disulphide-sulphur accounting for a 4.7% cleavage of the injected aminoethylcysteine to cystamine has been detected in the 24 h urine of rats after the injection. Paper and ion-exchange chromatography of the same urines showed the presence of unusual ninhydrin-reacting sulphur-containing compounds. Among them the following compounds have been tentatively identified: aminoethyl-(α,N-acetyl)-cysteine, unchanged aminoethylcysteine, lanthionamine, cystamine in a conjugated form. Cystamine has been extracted from the hydrolyzed urines and shown to be identical to a synthetic sample by chromatographic migration in different solvents and by paper electrophoresis.

RÉSUMÉ

Des rats albinos ont été injectés avec une solution de S-aminoéthyl-cystéine synthétique et les urines ont été analysées pour la détermination du soufre-disulfure total par la méthode de FOLIN-MARENZI et du soufre sous forme de cystine par la méthode de SULLIVAN ET HESS. Dans l'urine de 24 heures de rat injecté avec l'aminoéthylcystéine on a décelé une quantité de soufre-disulfure ne se trouvant pas sous forme de cystine qui témoignerait d'une scission du 4.7% de l'aminoéthyl-

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cystéine injectée en cystamine. La chromatographie sur papier et par échangeur d'ions des mêmes urines comparées avec celle des urines de rat au régime de base a mis en évidence la présence d'un certain nombre de composés nouveaux réagissant soit avec la ninhydrine soit avec les réactifs du soufre. Parmi eux les composés suivants ont été identifiés provisoirement: aminoéthyl-(α, N-acétyl)-cystéine, aminoéthyl-cystéine immodifiée, lanthionamine et cystamine sous une forme conjuguée. La cystamine a été extraite des urines hydrolysées et son identité avec la cystamine synthétique a été démontrée par migration chromatographique avec différents solvants et par électrophorèse sur papier.

ZUSAMMENFASSUNG

Weissen Ratten wurde synthetisches S-Aminoäthylcystein injiziert und im Harn wurde der totale Disulfidschwefel nach der FOLIN MARENZI-Methode und der Cystinschwefel nach der Methode von SULLIVAN UND HESS bestimmt. Im Harn von 24 Stunden nach der Injektion wurde eine Menge von nicht-Cystin Disulfidschwefel gefunden, die einer 4.7 %-igen Spaltung des injizierten Aminoäthylcysteins in Cystamin entspricht. Papier- und Jonenaustausch-Chromatographie desselben Harns zeigten die Anwesenheit einiger seltenen, schwefelhaltigen, Ninhydrin-positiven Verbindungen. Unter ihnen wurden die Folgenden vorläufig identifiziert: Aminoäthyl-(α, N-acetyl)-cystein, unverändertes Aminoäthylcystein, Lanthionamin und Cystamin in einer konjugierten Form. Das Cystamin wurde aus dem Harnhydrolysat extrahiert und seine Identität mit dem synthetischen Cystamin wurde durch Chromatographie in verschiedenen Lösungsmitteln wie auch durch Papierelektrophorese bewiesen.

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