RADIOAUTOGRAPHIC DETECTION OF METABOLITES OF ^{35}S-DL-CYSTINE

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As part of a general program of investigation of the metabolism of cystine in mammals, this paper presents the results of experiments made using labeled cystine in the rat. ARNSTEIN AND CRAWHALL[1] and AWAPARA[2,3] were the first to use labeled cystine for this type of investigation. In the present paper we describe the occurrence of a number of unsuspected cystine metabolites as detected by paper radioautography of the kidney extract and the urine of the rat injected with labeled cystine. A preliminary identification of some of the detected compounds has been possible by using the criterion of identity based on the fingerprint-like comparison between the radioactive spot of the unknown and the ninhydrin spot of the authentic sample of the suspected product added on the same chromatogram.

METHODS AND MATERIALS

^{35}S-DL-cystine was obtained from Amersham, England. It had an activity of 1 mC/6.9 mg. 2 mC were dissolved in 0.3 ml 1 *N* HCl, diluted to 2 ml with water, divided into two portions and injected intravenously into two male rats weighing 200 g. One rat was killed after 2 h, the kidneys were removed and an extract suitable for chromatography was obtained as described by AWAPARA[4]. The final aqueous extract (2 ml) was stored in the deep freeze. The other rat was kept in a metabolic cage and the urine of 24 h was collected under toluene. The sample was diluted to 30 ml with the washings of the funnel and was stored in the deep freeze.

Chromatograms were made on Whatman No. 4 paper using water-saturated phenol in the first direction and a mixture of collidine and lutidine saturated with one volume of water, in the second direction. Radioautograms were made by lightly pressing the dried chromatograms on a 30 × 40 cm X-ray film in the dark for a suitable length of time (10–20 days). After radioautography, the same chromatograms were developed with ninhydrin in order to locate amino acids and other ninhydrin-reacting compounds.

RESULTS

The radioautogram of 0.1 ml of kidney extract from the rat injected with labeled cystine is reproduced in Fig. 1. A very large number of compounds appears on this autogram, at least 12 different radioactive spots have been enumerated. Among these only taurine and hypotaurine may be easily identified by their unequivocal chromatographic location. A few other spots have been identified after careful control with an authentic sample of the suspected compound. All the remaining products are unknown.

Fig. 2 shows the same chromatogram used for the radioautography of Fig. 1, after the reaction with ninhydrin. It can be seen that, with the exception of taurine, all the radioactive compounds are present in such low amount that they cannot be

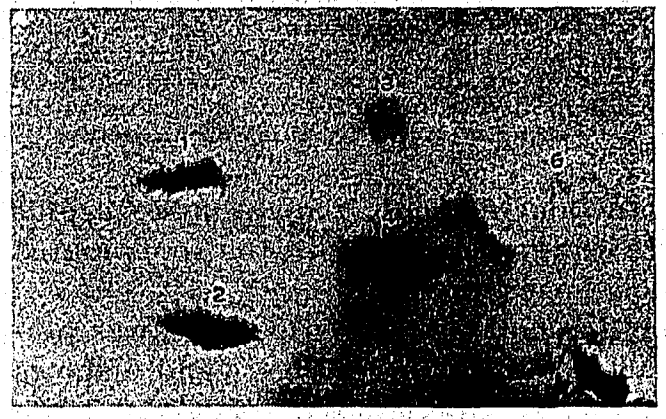

Fig. 1. Radioautogram (20 days) of 0.1 ml of kidney extract from a rat injected with 1 mC of ^{35}S-DL-cystine. Spots: 2 = hypotaurine; 4 = taurine. For the identification of the remaining spots see text. Sample applied at the right lower corner.

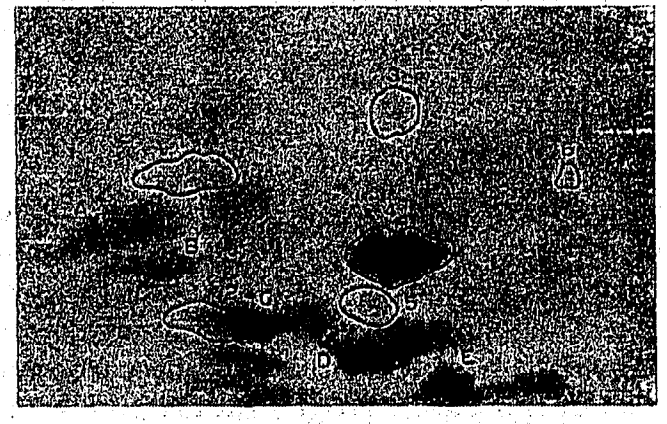

Fig. 2. The same chromatogram used for the radioautography of Fig. 1, after development with ninhydrin. The outlines of the more important radioactive spots seen in Fig. 1 have been marked in this chromatogram. Some of the ninhydrin-reacting spots have been identified as follows: A = leucines; B = valine; C = alanine; D = glycine; E = glutamic acid.

detected with ninhydrin, although a number of them certainly have a ninhydrin-reactive NH_2-group. In order to emphasize this point more strongly, the position of some of the radioactive spots seen in Fig. 1 have been marked by pen on the chromatogram of Fig. 2.

Very recently thiotaurine has been detected in the urine of rats fed with unlabeled cystine[5]. We deemed it of interest to confirm this finding using the labeled compound. The radioactive spot marked as No. 3 in Fig. 1 shows a chromatographic behavior like that expected for thiotaurine. In order to establish the identity of this spot with thiotaurine, a chromatogram was made with 0.2 ml of the kidney extract to which 50 μg of a pure sample of synthetic thiotaurine[6] had been added. After contact with the X-ray film for 10 days, the chromatogram was removed and developed with ninhydrin. Fig. 3 represents the radioautography of the same chromatogram developed with ninhydrin which is shown in Fig. 4. It can be seen that in the chromatogram the new spot of the added thiotaurine is now present, located in the same area as the

Fig. 3. Radioautogram (10 days) of 0.2 ml kidney extract from a rat injected with 1 mC ^{35}S-DL-cystine, to which 50 μg of unlabeled synthetic thiotaurine had been added.

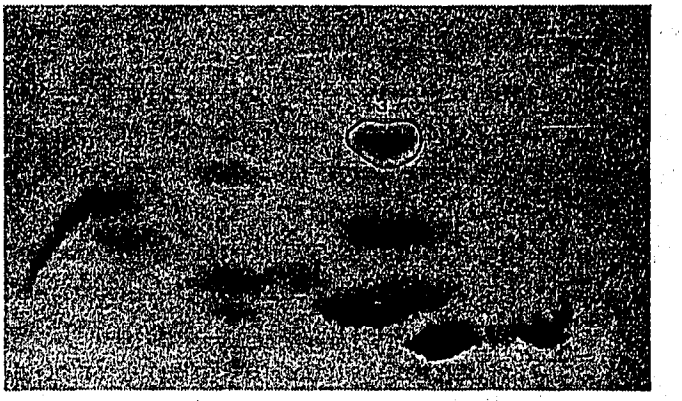

Fig. 4. The same chromatogram used for the radioautogram of Fig. 3, developed with ninhydrin. The spot marked No. 3, which is not seen in the chromatogram of Fig. 2, is due to the added thiotaurine. The identity in the position, in the shape and in every detail of this spot with the radioactive spot seen in the same area in Fig. 3, is emphasized.

radioactive spot No. 3. It may be further seen that not only the chromatographic coordinates of the two spots are identical but that the outlines of both the spots match perfectly well in every detail, like two identical fingerprints.

The same procedure was applied to spot No. 1 which, by its location, was presumed to be due to thiazolidine carboxylic acid. Comparison of Figs. 5 and 6 shows also in this case that the radioactive spot is perfectly superimposable in every detail on the ninhydrin spot of the same chromatogram made with added pure thiazolidine carboxylic acid.

The identification of spots No. 3 and No. 1 respectively as thiotaurine and thiazolidine carboxylic acid was further confirmed by their oxidation to taurine and

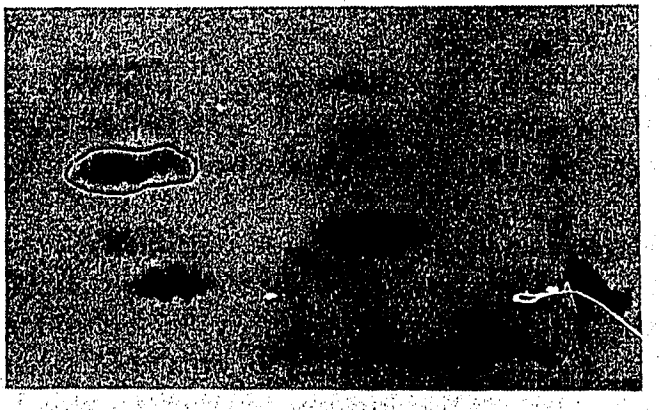

Fig. 5. Radioautogram (10 days) of 0.2 ml kidney extract from a rat injected with 1 mC ^{35}S-DL-cystine, to which 50 μg of unlabeled synthetic thiazolidine carboxylic acid had been added.

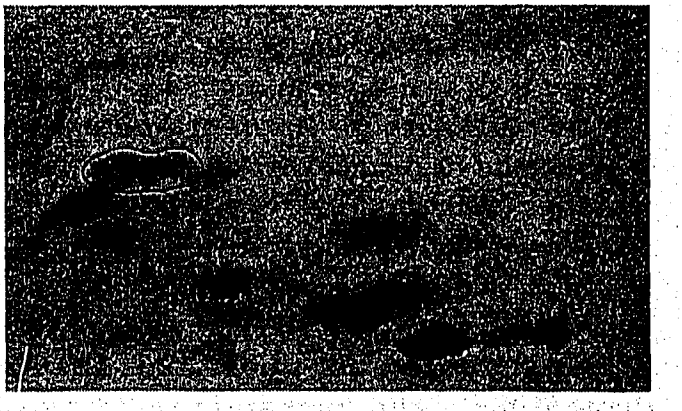

Fig. 6. The same chromatogram used for the radioautogram of Fig. 5, developed with ninhydrin. The spot marked No. 1 which is not seen in the chromatogram of Figs. 2 and 4 is due to the added thiazolidine carboxylic acid. The identity in the position, in the shape and in all details of this spot with the radioactive spot seen in the same area in Fig. 5, is emphasized.

to cysteic acid. Using a radioautogram as a guide, the areas of the two spots were cut out of the respective chromatograms. The fragments were eluted with water, the solution was treated with 1 ml 30% hydrogen peroxide in the presence of a trace of molybdate[7], and two new chromatograms and radioautograms were made with the final liquids. Among minor products, a spot of taurine and one of cysteic acid were detected as required for thiotaurine and thiazolidine carboxylic acid respectively.

Radioautography and chromatography of a 0.2 ml sample of the urine of the second rat are shown in Figs. 7 and 8. The chromatographed sample has been added with 50 μg of a pure sample of cysteinsulfonate[8] in order to show, by the same criterion of identification used above, the identity of spot No. 6 with cysteinesulfonate. The

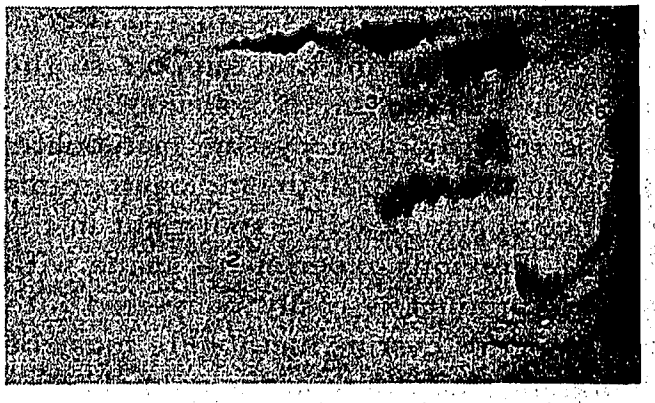

Fig. 7. Radioautogram (14 days) of 0.2 ml urine of rat injected with 1 mC ^{35}S-DL-cystine, added with 50 μg of synthetic unlabeled cysteinesulfonate. Spots: 2 = hypotaurine; 3 = thiotaurine; 4 = taurine; 6 = cysteinesulfonate.

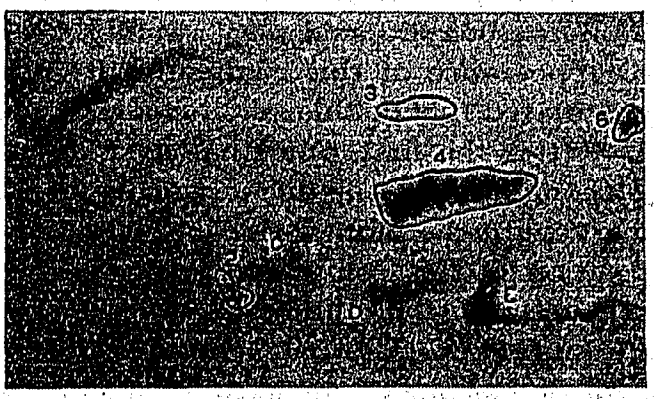

Fig. 8. The same chromatogram used for the radioautography of Fig. 7, developed with ninhydrin. The outline of some radioactive spots seen in Fig. 7 have been marked in this chromatogram. Some of the ninhydrin-reacting spots have been identified as follows: C = alanine; D = glycine; E = glutamic acid.

chromatogram of the urine without the addition of cysteinesulfonate obviously lacks spot No. 6.

The radioautogram of the urine is different from that of the kidney extract. Three very strong unidentified new spots appear which are not seen in the kidney extract. Moreover the spots identified in the kidney extract, namely, taurine, hypotaurine, thiotaurine, and thiazolidine carboxylic acid, are much weaker in the urine, probably owing to their higher dilution. Cysteinesulfonate has been detected in the urine; it is likely that the small spot falling in the same area in the autograms of kidney extracts (spots No. 6 of Figs. 1 and 2) is cysteinesulfonate itself.

DISCUSSION

Some of the metabolites of DL-cystine detected on the radioautograms have been identified. Among them are those present in larger amount. It is possible that some of the spots may be due to an artifact or may have originated from the unnatural portion of the racemic cysteine injected. Investigation is being continued in order to elucidate these points. Apart from this limitation, the large number of the metabolites

detected indicates the occurrence in the animal body of a number of metabolic pathways of DL-cystine yet to be discovered.

The identification of thiotaurine among the detected metabolites is of particular interest. It provides a confirmation of a previous finding and at the same time it serves to exclude the possibility that the thiotaurine first identified in the urine of cystine-fed rats[5] had a bacterial origin. Indeed, in the above experiments, cystine had been injected in the veins and thiotaurine was detected in the kidney extract, thus making any interference of the intestinal bacteria very unlikely.

The occurrence of thiazolidine carboxylic acid is another point which deserves attention. This product arises spontaneously by the interaction of cysteine with formaldehyde[9]. Enzymic systems have recently been described which oxidize thiazolidine carboxylic acid very rapidly to diformylcystine and other products[10, 11]. The disappearance of this compound from the urine gives experimental support to the postulated role of thiazolidine carboxylic acid in the metabolism of cysteine[10].

It is the first time that cysteinesulfonate is reported as a cystine metabolite. Although it has been unequivocally identified only in the urine, all the radioautograms made with the kidney extract show the occurrence of a radioactive spot right in the position of this compound (spot No. 6 of Fig. 1). Previous experiments made with synthetic cysteinesulfonate have shown that this product is easily converted into thiosulfate by the rat[12]; accordingly it is likely that this cystine metabolite must be regarded as one of the natural precursors of inorganic thiosulfate in the animal body.

This work forms part of a research program on the biochemistry of sulfur, sponsored by the Comitato Nazionale Ricerche Nucleari.

SUMMARY

Metabolites of ^{35}S-DL-cystine have been detected in the kidney extract and in the urine of rats by means of radioautography. Comparison of the chromatograms containing added pure samples of the suspected radioactive spots with their radioautograms permitted the identification of some of the detected compounds. The following cystine metabolites have been identified: taurine, hypotaurine, thiotaurine, thiazolidine carboxylic acid, cysteinesulfonate.

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