THE PATHOGENESIS OF CYSTINURIA. I. CHROMATOGRAPHIC AND MICROBIOLOGICAL STUDIES OF THE METABOLISM OF SULPHUR-CONTAINING AMINO-ACIDS

By C. E. DENT, J. G. HEATHCOTE,¹ and G. E. JORON ²

(From the Medical Unit, University College Hospital Medical School, London, and Research Association of British Flour-Millers, St. Albans, England)

(Submitted for publication March 16, 1954; accepted May 14, 1954)

Recent studies of cystinuria from this laboratory (1) have suggested that the disease does not, as had been assumed by most previous workers, involve an innate inability to metabolize one or other of the sulphur-containing amino-acids. On the contrary the defect responsible for the life-long constant excretion of large amounts of cystine appears to be due to a peculiarity only of renal function, that is, to a "low renal threshold" for cystine presumably due to diminished tubular reabsorption. This view was based in the first instance on the finding of large quantities of lysine, arginine, and perhaps ornithine, in the urine, a fact not readily explainable by Garrod's simple concept of an "inborn error of metabolism" of cystine, but quite consistent with the findings in certain other renal amino-acidurias. It was further supported by preliminary chromatographic and microbiological determinations of plasma cystine levels, since they were found to be no higher in cystinuric subjects than in normal subjects. This has since been confirmed by polarographic determinations (2). The renal theory had already been suggested by other workers but had only been subjected to one earlier incomplete investigation, which appears to have been largely overlooked (3).

Although the renal theory explains readily all of the spontaneously occurring clinical and biochemical phenomena it is still difficult to fit into it much of the work of others in which the metabolism of sulphur-containing amino-acids had been investigated (4). This work is often contradictory and suffers greatly from the inadequacy of the methods of analysis then available. The weight of evidence however indicated that cystine given by mouth to a cystinuric subject did not result in an increased excretion of cystine. Methionine and cysteine on the other hand did increase the cystine output. This had been interpreted as an error of metabolism not of cystine, but of cysteine, methionine acting only as a source of cystine (4).

In the present paper we report fuller investigations on the changes in plasma and urine amino-acids in two normal patients and two cystinuric patients after feeding loading doses of cystine, cysteine, and methionine. Only acute experiments on fasting subjects were carried out as this technique should exaggerate the metabolic changes more than the experiments done by others on the basis of 24-hour urine collections. Paper chromatography was chosen as the chief method of analysis owing primarily to its high specificity but also because of its ability to indicate changes in concentration of every amino-acid present in appreciable quantity, the importance of doing which having already been stressed (5). Owing to the only semi-quantitative nature, however, of paper chromatography, microbiological assays for cystine were also carried out in most of the experiments. As this quantitative method is also subject to question, especially in the case of cystine, the present report was held up until a more extensive check had been carried out by polarography (6).

METHODS USED AND CASES STUDIED

Cases. The experiments were performed on two normal subjects (G. J. and D. F.) and on two cystinuric subjects (L. L. and J. H.). G. J. was a male age 33, weight 81.8 Kg., D. F., a female age 23, weight 52.3 Kg. The cystinuric subjects (under the same initials) are described in a little more detail by Dent and Rose (1). Briefly, L. L. was a taxi driver aged 41 (weight 73 Kg.) who had had attacks of renal colic since 17 years of age, and had occasionally passed small smooth greyish white stones proved on analysis to be of cystine. X-ray showed a small opacity to be present in the left renal area. J. H. was a fitter aged 23 (weight 68 Kg.) who had had three attacks of renal colic at 11, 19, and 22 years of age. There

¹ Now at Distillers Company (Biochemicals) Ltd., Speke, Liverpool.
² Nuffield Dominion Travelling Fellow. Now at Montreal General Hospital, Montreal, Canada.
was no X-ray evidence of stones. His urine usually de-
posited typical cystine crystals on standing. The urine of
each cystinuric subject gave the typical cystine-ly-
sine-arginine pattern on chromatographic analysis.

Procedure during experiment. The subjects were fed
separately each of the three amino-acids, at least two
days intervening between each test. The quantities taken
were equivalent in respect of their sulphur content, i.e.,
5 g. of L-cystine, 6.2 g. of DL-methionine and 6.5 g. of
L-cysteine hydrochloride. The latter was neutralized
with NaOH to pH 7.0 immediately before ingestion.
The subjects ate nothing from the previous evening and until
the experiment was over. They were kept lying on a
couch throughout the test in order to minimize the changes
in glomerular filtration rate known to occur with changes in
posture and activity. At the beginning of test (0 hour)
the amino-acid was drunk either dissolved or suspended
in water, and a liberal water intake was maintained subse-
sequently to ensure adequate urine flow and bladder empty-
ing. Blood samples were obtained immediately before the
amino-acid was taken and again at ½, 1, 2, 4, and 6 hour
intervals. The blood was taken into a heparinized syringe,
immediately centrifuged and the plasma separated and
stored at 4°. Specimens were rejected if any trace of
hemolysis was present. The urine specimens were col-
lected at 0, 1, 3, 5, and 7 hours. The last time of emptying
the bladder before the experiment was also noted so that
the specimen collected at 0 hour could also be timed. All
the urines were preserved with thymol at 4°.

The normal subject, D. F., felt nauseated shortly after
swallowing the cystine and vomited shortly before the
1 hour blood sample was taken.

Chromatographic analysis. The plasma samples were
prepared for analysis by first, ultrafiltration to remove the
proteins, the method used being that described by
Dent and Schilling (7), secondly, by electrolytic de-
salting, using a micromodification (8) of the ingenious
apparatus devised by Consdon, Gordon, and Martin (9).
The urine samples were run without any pretreatment,
the volume taken for each analysis being that passed in
a given time interval, usually 2 sec. or 2/5 sec.

Two-way paper chromatograms (18 by 22 inches) were
used exclusively for the amino-acid analyses. Phenol/
NH4 and collidine-lutidine/EtOH-NH4 were the two solvents
used (10). Each sample of desalted plasma ultra-filtrate
(625 μl) was run alone and again after oxidation with
10 μl. H2O2 (30 per cent) and 10 μl. 0.1 per cent ammonium
molybdate so as to enable methionine and cystine (and/or
cystine) to be detected in the form of methionine sul-
phate and cysteic acid, respectively (11).

Each set of 5 to 6 samples of urine or of desalted ultra-
filtered plasma from one experiment was run simultane-
ously in the chromatogram cabinet. The resulting chrom-
atograms were strictly comparable with each other and
on being laid out side by side a most accurate picture of
the changes taking place in plasma amino-acid levels
and urine outputs could be obtained by merely comparing
in turn the strengths and sizes of each amino-acid spot
as it appeared in successive chromatograms. In order
to determine approximately the plasma cystine (and/or
cystine) concentration a known but varying quantity of
cysteic acid was placed on each chromatogram about 4
cm. from the site of the plasma or urine sample. The
quantities chosen covered the full range of cystine levels
anticipated in the samples and were as far as possible
placed on chromatograms likely to contain similar
amounts in the sample. In this way each cysteic acid
spot obtained from the oxidized cystine in the sample
could be matched by eye with a similar strength spot on
the same chromatogram or on one of the others in the
same batch, and a semi-quantitative estimate obtained.
This method was considered to be very accurate when
used for determining relative strengths of an amino-
acid in a series of samples from one experiment, and we
were impressed by the definiteness of the serial changes
shown as varying strengths of the spot on the chromato-
grams in, say, the cystine levels of plasma after feeding
cystine (see later). However, a fairly constant loss of
cystine always occurs during the H2O2 oxidation so we
expected the absolute values to be on the low side.

As a further check, each urine sample was tested directly
under standard conditions, for cystine and for cysteine by
the cyanide/nitro-prusside test (12).

Microbiological assays. Plasma samples were first pre-
pared for assay as previously described for chromato-
graphic analysis, except that the electrolytic desalting
was not carried out since preliminary experiments showed
that the small amounts of mercury introduced into the
samples proved to be toxic to the assay organism. After
ultrafiltration, each plasma sample was diluted, where
necessary, with distilled water to the appropriate volume
before assay for cystine at 2 to 4 levels in duplicate where-
ever possible.

The cystine assays were carried out using Leuconostoc
mesenteroides P-60 since, according to Riesen, Spengler,
Robblee, Hanes, and Elvejem (13), this organism is
the most specific in its response to cystine as distinct from
its simple derivatives, and the standard curves are more
reproducible than those obtained with any other strain
of bacteria. The most suitable medium was found to be
an oxidized peptone medium which was essentially that
described by Lyman, Moseley, Wood, and Hale (14) with
minor modifications chiefly in respect of the mineral and
vitamin concentrations.

Urine samples were diluted from 1:25 to 1:500 (by vol.)
depending on the concentration of cystine in the
urine. In some samples containing very high concentra-
tions, crystallization of the cystine had taken place be-
fore they were received for assay, and in such cases, care-
ful homogenization was necessary to ensure reproducible
results. The diluted urine samples were assayed in duplica-
te at 3 to 5 levels.

The microbiological assay of the true free cystine in
urine normally presents considerable difficulty owing to
the presence of many diverse substances stimulating to
the growth of the organism. The problem was overcome
in the present experiments in the following manner: A
solution was made by dissolving Difco peptone (50 g.) in
a mixture of urine (90 ml.), water (360 ml.) and concentrated HCl (50 ml.). To this solution, hydrogen peroxide (15 ml. of 100 vols.) was added and the whole was allowed to stand overnight. The mixture was then treated in the usual manner as described by Lyman, Moseley, Wood, and Hale (14) and diluted to one liter. The requisite salts, essential vitamins, methionine, tyrosine, and tryptophan were added to 400 ml. of this solution to make one liter, of double strength medium. The assay was carried out in the same manner and using the same organism as for the plasma assays. The sensitivity of the organism to cystine was increased considerably in this unusual medium and the assay was much improved. In addition, wherever possible, in the making up of the peptone-urine medium, the urine from the particular subject under investigation, whether control subject or cystinuric subject, was used. For this purpose, a separate, bulked, 24-hour sample of urine was collected before the feeding experiments were carried out.

Despite the precautions taken, the assays of both plasma and urine samples still left something to be desired, partly no doubt due to the presence of peptides in the former samples and partly due to the peculiar assay difficulties in the case of cystine referred to previously by one of us (15). Furthermore, when the present work was carried out, the improved procedure of Camien and Dunn (16) had not been published and it would seem likely, therefore, in view of these authors' results, that the present microbiological assay results for both plasma and urine are relatively high. Nevertheless the results were reproducible and the general trends reliable.

RESULTS

Chromatographic assays

The relative changes in plasma level and urinary output of cystine following the ingestion of the three amino-acids can be seen from the graphs in Figure 1.

These changes and those in plasma levels of other amino-acids were as follows:

(a) In no subject was the plasma cystine appreciably raised after giving the cystine. Following its ingestion there was a slight increase in \(\alpha\)-amino-\(n\)-butyric acid level in the earlier samples from the normal subject, D. F. The cystinuric subject, J. H., showed a slightly higher than normal concentration of \(\alpha\)-amino-\(n\)-butyric acid in all specimens obtained in the fasting state and following the ingestion of cystine there was a further slight increase in \(\alpha\)-amino-\(n\)-butyric acid and methionine which was maximal at 1 hour. These changes were not reflected in the urine outputs.

(b) Following the ingestion of methionine the plasma cystine level showed no consistent changes, but the methionine rose to a high level maximal at 1 to 2 hours, and remained still appreciably raised after 6 hours. The normal subjects and cystinuric subjects behaved similarly and in all cases there was also a definite slight rise in \(\alpha\)-amino-\(n\)-butyric acid level which was usually maximal in the 6-hour specimen. The urine output of methionine was greatly increased in all specimens passed while the plasma level was raised.

(c) Following the ingestion of cysteine there were in all cases large rises in plasma cystine levels.
The only other change of note was in the cystinuric subject, J. H., who (as after taking cystine) showed a slight rise in α-amino-γ-butyric acid level.

An important negative finding was the absence of any detectable changes, in any of the experiments, in the plasma level or urinary output of lysine and of arginine.

Twenty-four hour samples of urine of the cystinuric subjects J. H. and L. L. were collected while they were on the ordinary hospital diet. Chromatographic analysis of the cystine output gave in both cases figures of the order of 800 to 900 mg. per 24 hour.

The cyanide/nitro-prusside testing of all the urines gave colour reactions for the cysteine which corresponded approximately to the concentrations expected on the basis of the chromatographic assay. The nitro-prusside test also showed that traces of cysteine were present in some of the urines passed at the time when the maximal additional cystinuria was taking place after ingesting cysteine. At other times the urinary amino-acid appeared to be entirely cystine and not cysteine.

Microbiological assays

The results are shown in Figure 2 and Tables I–III. These confirm the main chromatographic findings as regards the changes in plasma cystine level and urine output (see above).

DISCUSSION

After ingesting a large dose (5 g.) of cystine, only slight changes in plasma cystine level occurred. This confirms Hier (17), who used microbiological assays, and Brown and Lewis (3), who used a chemical method. We also confirmed that no increased cystine output occurred in either normal or cystinuric subjects. After methionine, the results were more ambiguous. The plasma cystine levels probably rose slightly and this was definitely followed in the cystinuric patients by an increased cystine output although the change in the normals, if any indeed occurred, was too small to be detectable. Spectacular changes however occurred after feeding cystine. In all four subjects the plasma cystine level rose considerably and was associated with an increased urinary output. We appear, therefore, to have a ready explanation of the results of the metabolic experiments done by earlier workers, namely, the cystine output in the urine in any given subject is dependent mainly, at least, on the plasma cystine level, and the extent of cystine output at a given plasma level depends on whether the patient is cystinuric or otherwise, as the cystinuric patients always leak out into their
urine more cystine than the normal subjects at all plasma cystine levels. This finding supports the "low renal threshold" theory of Dent and Rose (1) and is strong evidence against the "error of metabolism" theory of Garrod (18) and others. The equally rapid fall in the high plasma cystine levels shown by both normal and cystinuric subjects after taking cystine is an important piece of evidence against the latter theory. If a true "error of metabolism" were present a "diabetic" type of cystine tolerance curve would have been expected in the cystinuric subjects.

Our results however still leave some puzzling features inadequately explained. Why does the plasma cystine level rise so much more after giving cysteine than after cystine? Brand, Cahill, and Harris (4) sought to explain the results of their metabolic experiments (in which the urine only was examined) by postulating that there were different metabolic pathways for cystine and cysteine and that it was only the metabolism of the cystine (which was chiefly derived from methionine) that was disturbed in cystinuric subjects. We find this suggestion difficult to accept in view of the ready interconvertibility that must exist in the body between \(-S-S-\) and \(-SH\) groups whether in the form of free amino-acids, peptides, such as glutathione, or whole proteins. Moreover in our results we have found no essential differences between the way normal and cystinuric subjects metabolize these amino-acids. Our tentative hypothesis is, therefore, that the differences in plasma levels may be due to different rates of absorption from the gut. Cysteine being readily soluble in water is very rapidly absorbed through the intestinal mucous membrane, probably all within the first hour, and it therefore reaches the portal blood in such a high concentration as temporarily to saturate the mechanisms for maintaining a constant blood level of cystine. The large dose of cystine on the other hand remaining almost entirely undissolved in the intestinal fluids is only taken into the portal blood relatively slowly and so is adequately dealt with by the liver and never reaches the systemic blood in appreciably raised concentrations. Another possibility is that the cystine is partly decomposed, say, into \(\text{H}_2\text{S}\) and alanine, by bacteria while in the gut, so preventing some from being absorbed at all. Our experiments with cystine were sufficiently prolonged for maximal absorption since earlier work on the increased urinary sulphur output in the urine after giving cystine by mouth has shown that the cystine must have been largely absorbed in some form or other in the 6-hour space of our experiment.

It has been repeatedly found in rats (Dent and Harbury, Unpublished work) that a similar cystinuria can be induced by feeding a single large dose of cysteine but not by the same dose of cystine. So, whatever the explanation, we are convinced that this is a general phenomenon which is not confined to cystinuric subjects. We can only find one reference in the literature to the feeding of cysteine to normal humans namely, Hess and Sullivan (19) who gave 2.6 g. of the hydrochloride but reported no increased output of cystine in the 24-hour urine. Our present results do not confirm this and provide evidence that there is probably no clear-cut difference between the metabolism of cysteine by normal and cystinuric subjects. Increased cystine output after cysteine feeding cannot therefore be used as a diagnostic test for cystinuria (Freudenberg [20]).

**SUMMARY**

1. Chromatographic analyses of plasma and urine have been carried out during the 6 hours following the ingestion of large single doses of cyst-
Pathogenesis of Cystinuria

tine, cysteine, and methionine by two normal and two cystinuric subjects. Most of the samples were also assayed by microbiological methods.

2. The changes observed showed similar trends in both normal and cystinuric subjects.

3. Following ingestion of cystine only slight, possibly insignificant, changes in plasma cystine level were detected. There was no change in urinary cystine output.

4. Following ingestion of methionine there were only slight rises in plasma cystine levels but a slight increase in urinary cystine output occurred in the two cystinuric subjects. The plasma contained very high concentrations of methionine.

5. Following ingestion of cysteine there were very large rises in plasma cystine levels. Greatly increased urinary cystine outputs were also observed in both normal and cystinuric subjects.

6. The changes in output of cystine in the urine in any given subject roughly followed changes in plasma cystine level. In the two cystinuric patients, however, the urinary output was greatly increased over normal at all plasma levels.

7. These results are taken to support the suggestion of Dent and Rose (1) that in cystinuria the body tissues as a whole show no "error of metabolism" for cystine in the sense in which this term was used by Garrod. The biochemical findings in cystinuria can be better explained on the basis only of kidney tubule dysfunction.

8. Consistent slight changes occurred in the levels of α-amino-n-butyr acid in some of these experiments, as if to suggest that it is closely related metabolically to the sulphur containing amino-acids (see also Dent and Schilling [7]).

REFERENCES