

## SUMMARY

1. Compounds containing *N*-terminal residues have been shown to be present in preparations of soluble collagen which account for most if not all the reaction of fluorodinitrobenzene with  $\alpha$ -amino groups.

2. The significance of the residues has been discussed in relation to the known molecular weight of collagen as determined by physical means. The terminal residues are thought to be due to non-protein nitrogen firmly attached to collagen and not to belong to collagen as true *N*-terminal residues in the usually accepted meaning of the term '*N*-terminal residue'.

3. The thermal conversion of collagen into gelatin has been shown to take place as a physical dissociation with the minimal rupture of peptide bonds.

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## The Degradative Metabolism of L-Cysteine and L-Cystine *in vitro* by Liver in Cystinosis

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Since Abderhalden (1903) first detected widespread storage of crystalline cystine in the tissues of an infant at autopsy, and noted the familial incidence of the disorder, an increasing number of cases have been reported in which cystinosis has been diagnosed by post- and ante-mortem examination (Lignac, 1924; McCune, Mason & Clarke, 1943; Worthen & Good, 1958). The associated clinical features of this disease include vitamin D-resistant rickets, glucosuria, generalized hyper-amino aciduria, and acidosis, symptoms which characterize a group of diseases based on the failure of reabsorptive and base-conserving function of the renal tubules, and often referred to as the Fanconi syndrome (Fanconi, 1936). Several hypotheses seeking to explain cystine storage and to relate this to the Fanconi syndrome have been put forward. Bickel *et al.* (1953) reported increased concentrations of a number of plasma amino acids in cystinosis, and suggested that these resulted

from a generalized failure of their normal incorporation into proteins. The intracellular deposition of the poorly soluble cystine and the renal anomalies were considered to be manifestations of this primary defect. However, essentially normal plasma amino acid concentrations have been found by several authors using chromatographic methods (Dent, 1947; Evered, 1956; Brigham, Stein & Moore, 1960), and these are now generally accepted as proof that the amino aciduria is of renal origin.

A second suggestion proposes a specific enzymic defect in cysteine-cystine metabolism, leading to storage of the resultant excess of cystine in the tissues and consequent nephrotoxic effects. The reduction of cystine to cysteine by cystine reductase has been considered the most probable site of such a defect (see Worthen & Good, 1958). Since no investigation of these latter proposals had been reported, a comparison of cystinotic- and normal-

liver preparations, with respect to the degradation of cysteine and cystine, was undertaken. Particular attention has been given to the enzymic reduction of cystine.

Recently, deficiencies in the reduction of cystine by two different enzyme systems of blood have been reported in cases of cystinosis. Worthen & Good (1961) concluded that cystine reductase (NADH<sub>2</sub>-L-cystine oxidoreductase, EC 1.6.4.1) was deficient in two cases examined, whereas Seegmiller & Howell (1961) reported cases of low activity of a system catalysing the transfer of hydrogen from reduced glutathione to cystine. Neither of these discordant results is supported by the present study.

### MATERIALS

*Autopsy specimens.* The following cases of cystinosis were studied: Case 1, female, aged 11 yr.; Case 2, male, aged 8 yr.; Case 3, female, aged 7 yr. Tissues for comparison were obtained from two cases of congenital heart disease in which no primary metabolic abnormality had been apparent. These are referred to as Normal 1 (female, aged 8 yr.) and Normal 2 (male, aged 6 yr.). Autopsies were performed within 2 hr. after death. Portions of liver and kidney were placed in polyethylene bags and frozen immediately in crushed solid CO<sub>2</sub>. Thereafter, specimens were stored at -25°. Only small samples of cystinotic kidney were obtained, their use being limited to tests for cystine reduction.

*Chemicals.* The following chemicals were obtained from the sources indicated: NADH<sub>2</sub> (disodium salt), NADP (monosodium salt), pyridoxal phosphate and  $\alpha$ -oxoglutaric acid, from the Sigma Chemical Co.; glucose 6-phosphate (disodium salt), NADPH<sub>2</sub> (monosodium salt) and L-glutamic acid, from the British Drug Houses Ltd.; L-cystine, L-cysteine, L-cystic acid, reduced glutathione (GSH) and NAD from L. Light and Co. Ltd.; L-cysteine-sulphinic acid, from the California Corp. for Biochemical Research, Los Angeles. All other reagents were of AnalaR grade where obtainable.

### METHODS

#### *Preparation of acetone-dried liver powder*

Chopped frozen liver (10 g.) was homogenized by grinding with sand and water (20 ml.) at 0°. After standing in the cold for 1 hr. with occasional stirring, the suspension was filtered through surgical gauze, and acetone (2 vol.) at -5° was added to the filtrate with continuous stirring. The precipitate was collected by rapid filtration in the cold, and washed with cold acetone and finally with ether. The powders were stored *in vacuo* over magnesium perchlorate and liquid paraffin.

#### *Tissue homogenates and slices*

Homogenates (usually 20%) were prepared in the appropriate ice-cold media with a Potter-type glass homogenizer for 1 min. at about 700 rev./min. The layer of fat obtained from certain specimens after centrifuging at 800g for 15 min. at 2° could be removed almost completely by screening through several layers of surgical gauze.

Slices were cut free-hand from frozen blocks, and suspended in the cold buffer to be used in the particular assay. Suitable slices, selected for translucency, were collected into bundles and blotted before transfer to incubation flasks. In slice experiments, tissue-dry weights from individual flasks were determined at the end of the incubation period by removal of the total contents (washing with water if necessary to remove small fragments), to which was then added an equal volume of 10% (w/v) trichloroacetic acid. After centrifuging, and washing with water and with acetone, the residues were dried to constant weight at 100°. Homogenate (0.5 ml.) was precipitated with trichloroacetic acid, and washed and dried similarly. The protein content of other enzyme solutions was measured spectrophotometrically (Warburg & Christian, 1941).

#### *Enzyme preparations*

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP oxidoreductase, EC 1.1.1.49) was prepared from yeast by the method of Kornberg (1950), and assayed according to Kornberg & Horecker (1955). The final solution had an activity of 30 units/ml. Yeast glutathione reductase [NAD(P)H<sub>2</sub>-glutathione oxidoreductase, EC 1.6.4.2] was prepared and assayed according to Racker (1955). The preparation used was that obtained at the first ethanol-precipitation stage, and possessed an activity of approximately 20 000 units/ml. Oxidized glutathione (GSSG) used in the reductase assay was prepared by the method of Rall & Lehninger (1952).

#### *Enzyme assays*

*Cystine reductase.* The activity of cystine reductase in acetone-dried liver powders was estimated by the initial decrease in NADH<sub>2</sub> extinction at 340 m $\mu$  in the presence of cystine added as a saturated solution in either 0.02M-phosphate buffer, pH 6.5, or 0.1M-tris buffer, pH 8.2. Under these conditions the solubility of cystine at 22° was 11.6 and 43.6 mg./100 ml. in the phosphate and tris buffers respectively, as determined by the method of Sullivan, Hess & Howard (1942). An extract of acetone-dried powder (0.3 g.) was prepared in 3 ml. of the appropriate buffer, and dialysed overnight in the cold against the same buffer. Clear extracts were obtained by centrifuging for 30 min. at 6000g. Kidney extracts were assayed similarly. Cystinotic kidney, owing to its fibrous nature, could not be homogenized satisfactorily in the Potter homogenizer. Extracts were prepared by grinding 1 g. with cold 0.02M-phosphate buffer (pH 6.5, 3 ml.) and sand. After dialysis for 40 hr. against this buffer, clear extracts were obtained by centrifuging at 3000g for 20 min.

Alternative methods of estimation were used for liver homogenates, which were dialysed in cellophane tubing (5 mm. diam.) for 40 hr. in the cold against the buffer to be used in the assay. In the first method, homogenate in phosphate buffer, pH 6.5, was shaken anaerobically in Warburg flasks for 2 hr. with NADH<sub>2</sub> and solid cystine, and the increase of thiol groups in samples deproteinized with 10% trichloroacetic acid (0.5 vol.) was determined by titration with 5 mN-iodine. The starch-iodine end point was detected on an EEL Micro-titrator (Evans Electro-selenium Ltd.). In the second method, homogenate was incubated in tris buffer, pH 8.5, containing NADH<sub>2</sub> and cystine, and NADH<sub>2</sub> extinctions at 340 m $\mu$  were measured



at zero time and after 10 min. with samples which had been deproteinized by the addition of 95% ethanol (1 vol.) followed by 10% (w/v) sodium sulphate (0.1 vol.). A 5 mM-cystine soln. used in these tests was prepared by dissolving L-cystine (30 mg. of free base) in N-sodium hydroxide (1 ml.) and adding 0.05M-tris, pH 8.5 (5 ml.); the solution was readjusted to pH 8.5 with N-hydrochloric acid, and diluted to 25 ml. with water. Solutions prepared in this way were stable for at least 4 hr.

*System reducing cystine in the presence of reduced glutathione.* Liver homogenate (20%) in 1.15% (w/v) potassium chloride was dialysed for 24 hr. in the cold against 0.1M-tris buffer, pH 7.6. The incubation medium, added to Warburg flasks, contained in a final volume of 5 ml.: 0.1M-tris, pH 7.6 (1 ml.); 0.5M-glucose 6-phosphate (0.2 ml.); 0.1M-magnesium chloride (0.2 ml.); glucose 6-phosphate dehydrogenase (10 units); 5 mM-NADP (0.1 ml.); 10 mM-GSH (0.2 ml.); mM-pyridoxal phosphate (0.1 ml.); homogenate (0.5 ml.); solid cystine (5 mg.). Side arms contained 0.2 ml. of a zinc acetate reagent (see below) to trap any hydrogen sulphide liberated. At zero time, a sample (2 ml.) of medium was deproteinized with 10% trichloroacetic acid (1 ml.), and the clear supernatant (2 ml.) obtained after centrifuging was titrated with 5 mM-iodine. Incubation flasks were flushed with nitrogen, and, after shaking for 2 hr. at 37°, the iodine titration for thiol groups was repeated. Trapped hydrogen sulphide was estimated as in the assay of cysteine desulphhydrase.

*Cysteine desulphhydrase.* The assay medium, consisting of 0.067M-phosphate buffer, pH 7.4 (1 ml.), liver homogenate, 20% in 0.9% sodium chloride, (1 ml.) and mM-pyridoxal phosphate (0.1 ml.), was added to the main compartments of Warburg flasks. Side arms contained 0.1M-cysteine, pH 7.4 (0.2 ml.), and centre wells a reagent (0.2 ml.) consisting of hydrated zinc acetate (6.0 g.), hydrated sodium acetate (1.7 g.) and sodium chloride (0.005 g.) dissolved in water (100 ml.). Flasks were flushed with nitrogen, and shaken for 90 min. at 37°. *p*-Amino-*NN*-dimethylaniline [0.3 ml. of a 0.05% soln. in 20% (w/v) sulphuric acid] followed by 1 drop of 10% (w/v) ferric chloride in 0.1N-hydrochloric acid was added to the centre well, and the resulting methylene blue solution, after colour development for 1 hr., was washed out and diluted to 25 ml. with water. Extinctions at 630 m $\mu$  were measured in 1 cm. cuvettes. An aq. hydrogen sulphide soln., standardized with sodium arsenite, was used to prepare a standard curve for the range 10–100  $\mu$ g. of hydrogen sulphide.

*Desulphurization of cystine.* This system refers to the NADH<sub>2</sub>-dependent reduction of cystine to cysteine, followed by the liberation of hydrogen sulphide by cysteine desulphhydrase [L-cysteine hydrogen sulphide-lyase (deaminating), EC 4.4.1.1]. The hydrogen sulphide, estimated as described above, gave a measure of the overall reaction. Alcohol dehydrogenase (alcohol-NAD oxido-reductase, EC 1.1.1.1), contained in the liver preparations, was used to regenerate NADH<sub>2</sub>. A 10% liver homogenate was prepared in 0.9% sodium chloride and dialysed overnight in the cold against 0.067M-phosphate buffer, pH 7.4.

*Decarboxylation of L-cysteate and L-cysteinesulphinate.* This was determined for liver extracts according to the method of Hope (1955).

*Oxidation of L-cysteine and L-cystine to inorganic sulphate.* Liver slices were suspended in 5 ml. of bicarbonate-saline (Krebs & Henseleit, 1932), in which magnesium sulphate

was replaced by magnesium chloride (Pirie, 1934). The Warburg flasks were flushed continuously with O<sub>2</sub>+CO<sub>2</sub> (19:1). At zero time 0.4M-cysteine (0.2 ml.), pH 7.4, was added from the side arms, and the flasks were shaken at 37°. When cystine was used as substrate this was added as the solid (5 mg.) directly to the main compartments at the beginning of the experiment. After 2 hr., inorganic sulphate in the deproteinized medium (i.e. after addition of 5 ml. of 10% trichloroacetic acid) was determined by a modification of the method of Cuthbertson and Tompsett (Maw, 1954). In later experiments the method of Dodgson (1961) was found to give more reproducible results.

*Oxidation of L-cysteinesulphinate.* The metabolism of L-cysteinesulphinate by liver slices in the presence of  $\alpha$ -oxoglutarate was measured in the following medium: 0.1M-phosphate, pH 7.4 (2 ml.); slices (approx. 100 mg. fresh wt.); 0.376M-L-cysteinesulphinate, pH 7.4 (0.2 ml.); water (2 ml.); added to Warburg flasks. The gas phase was air, and centre wells contained 10% (w/v) potassium hydroxide (0.2 ml.). After equilibration for 10 min. at 37°, 0.25M- $\alpha$ -oxoglutarate, pH 7.4 (0.2 ml.), was added from the side arm, and oxygen uptake followed for 2 hr. The flask contents were treated with cold 10% trichloroacetic acid (5 ml.), and, after centrifuging, pyruvate was estimated by the method of Friedemann & Haugen (1943). Inorganic sulphate was estimated according to Dodgson (1961). Glutamate was estimated chromatographically. Supernatant (2 ml.) was evaporated to dryness, and the residue was extracted with water (0.5 ml., or 0.1 ml. in experiments in which either  $\alpha$ -oxoglutarate or cysteine-sulphinate was omitted from the medium). Samples (30  $\mu$ l.) were chromatographed in butan-1-ol-acetic acid-water (12:3:5, by vol.) on Whatman no. 2 paper. Marker solutions containing 10, 20 and 30  $\mu$ g. of glutamate were run simultaneously. After spraying with ninhydrin, the approximate concentrations of glutamate in test solutions were estimated by visual comparison. This was found to give estimates in fair agreement with those obtained by spot elution and colour measurement.

*Glutathione reductase.* This was assayed essentially according to the method of Racker (1955). Acetone-dried liver powder (50 mg.) was extracted with cold water (1 ml.) for 1 hr. After centrifuging, the solution was dialysed overnight in the cold against 0.2M-phosphate, pH 7.6, and recentrifuged.

All spectrophotometric measurements were made in 1 cm. silica cells with a Unicam SP. 500 spectrophotometer (Unicam Instruments Ltd.).

## RESULTS

*The reduction of cystine by cystine reductase.* A comparison of the activities of cystine reductase in liver homogenates and kidney extracts, and in acetone-dried liver powders of the cystinotic and normal cases, is shown in Table 1. Tests were made after the tissue samples had been stored for 2 months at -25°, except for cystinosis Case 1 (Expt. D), which had been stored for 3 months. The activities of the cystinotic- and normal-tissue preparations were in good general agreement in each experimental series, with different conditions

Table 1. *Cystine-reductase activity of liver and kidney preparations*

Silica cuvettes of 1 cm. light path contained 2.5 ml. of a saturated soln. of cystine in 0.02M-phosphate buffer, pH 6.5 (Expt. A), or in 0.1M-tris buffer, pH 8.2 (Expt. B); acetone-dried liver-powder extract (0.5 ml.) (see Methods section); and 1.4 mM-NADH<sub>2</sub> (0.1 ml.). Extinctions at 340 m $\mu$  were measured at 1 min. intervals for 10 min. at room temperature (22°). Blank assays contained buffer instead of buffer-cystine soln. Kidney extract (0.5 ml.) (see Methods section) was assayed similarly in 0.02M-phosphate, pH 6.5 (Expt. C). In Expt. D, incubation media contained liver homogenate (1 ml.); 0.05M-tris buffer, pH 8.5 (1 ml.); 5 mM-cystine, pH 8.5 (2 ml.), or water; 3 mM-NADH<sub>2</sub> (0.2 ml.); in 5 ml. final volume. Incubation temperature was 30°. Extinctions at 340 m $\mu$  of samples deproteinized at zero time and after 10 min. (see Methods section) were measured. In Expt. E, media contained liver homogenate (2 ml.); 0.02M-phosphate, pH 6.5 (2 ml.); 14 mM-NADH<sub>2</sub> (0.1 ml.); water (1 ml.); solid cystine (5 mg.); added to Warburg flasks. Flasks were shaken anaerobically at 37° for 2 hr., and the increase of thiol groups was determined by titration with 5 mM-iodine.

Case	Activity ( $-\Delta E_{340}$ /mg. of protein/hr.): Acetone-dried-liver- powders preparation		Activity ( $-\Delta E_{340}$ /mg. dry wt./hr.)		Thiol groups formed ( $\mu$ moles/g. dry wt./hr.). Liver-homogenate preparation Expt. E
	Expt. A	Expt. B	Kidney- extract preparation Expt. C	Liver- homogenate preparation Expt. D	
Cystinosis 1	0.052	0.055	0.019	0.024	25.5
Cystinosis 2	0.039	0.031	0.021	0.029	33.0
Cystinosis 3	0.038	—	—	0.024	29.0
Normal 1	0.047	0.050	0.024	0.026	31.5
Normal 2	0.044	—	—	0.021	25.5

and methods of assay. It was found that the cystinotic homogenates released thiol groups in the absence of added cystine when such preparations were dialysed for only 24 hr. This activity, which was completely dependent on the addition of NADH<sub>2</sub>, in some homogenate preparations amounted to 60% of that obtained by full stimulation with added cystine. Dialysis for 40 hr. in cellophane tubing (5 mm. diam.) reduced this endogenous activity almost to zero without causing undue loss (not more than 10%) of overall cystine-stimulated activity. It appears certain from these experiments that the high endogenous activity was due to stored cystine, low residual concentrations of which would be sufficient to saturate the enzyme in the most active of the preparations tested. Fig. 1 shows the effect of varying concentrations of such a preparation in assay media containing the lowest concentrations of cystine, i.e. when cystine was added as a saturated solution, pH 6.5. The specific activities given in Table 1 were confined to determinations carried out in the linear range of the activity curves. Fig. 1 also illustrates the specificity of cystine reductase for NADH<sub>2</sub>, an equivalent amount of NADPH<sub>2</sub> failing to act as replacement, and shows the complete loss of activity of a boiled preparation.

Evidence that cystine-reductase activity is normal in cystinotic liver (stored frozen for 14 weeks) was also obtained from determinations of the desulphurization of cystine by homogenates. The liberation of hydrogen sulphide from cystine was completely dependent on additions of NAD and, with one exception (cystinosis Case 3, in

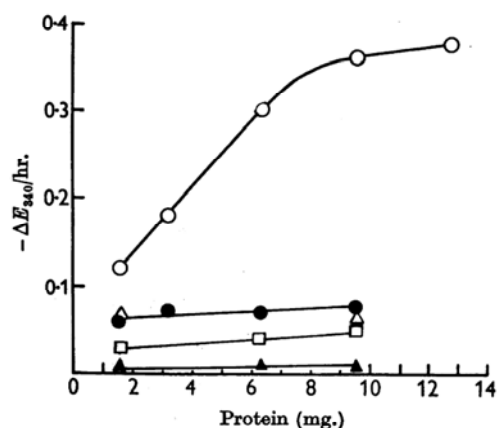


Fig. 1. Cystine-reductase activity of acetone-dried liver powder (cystinosis Case 3). Varying amounts of enzyme were assayed under the experimental conditions of Expt. A, Table 1. ○, Complete system; ●, cystine omitted; △, enzyme soln. boiled for 2 min.; □, NADH<sub>2</sub> replaced by NADPH<sub>2</sub>; ▲, NADH<sub>2</sub> omitted.

which a low endogenous activity was observed), a NAD-linked oxidizable substrate. In the experiments listed in Table 2, ethanol was used as oxidizable substrate for the regeneration of NADH<sub>2</sub> by alcohol dehydrogenase of the liver homogenates. The results are interpreted as showing a normal reduction of cystine to cysteine by cystine reductase of cystinotic liver, followed by the liberation of hydrogen sulphide from cysteine by cysteine desulphhydrase. The slight activity of



the cystinotic samples observed in the tests from which cystine was omitted was attributed to residual stored tissue cystine.

*The reduction of cystine by reduced glutathione.* In the presence of reduced glutathione, cystine is converted into cysteine. Liver homogenates stimulate this reaction, and further metabolize part of the cysteine formed, mainly through the desulphhydrase reaction under anaerobic conditions. With low concentrations of glutathione the overall system requires glutathione reductase, coupled with a reaction which regenerates NADPH<sub>2</sub>. In the experiments on liver homogenates, this latter requirement was met by the addition of the glucose 6-phosphate-dehydrogenase system. Homogenates possessed sufficient glutathione-reductase activity to maintain maximum concentrations of reduced glutathione throughout the incubation period. A comparison of the activities of glutathione reductase in cystinotic and normal liver shows good agreement of values independent of the period of storage of the frozen tissues (Table 3).

Table 4 shows the requirements of the cystine-reduced glutathione system for cystine, reduced glutathione, a NADP-linked oxidizable substrate (glucose 6-phosphate) and glutathione reductase. To account for the extent of spontaneous non-enzymic reduction of cystine by reduced glutathione in the overall reaction, it was necessary to employ a composite system in which a glutathione-reductase preparation was substituted for liver homogenate in these experiments. A comparable concentration of reduced glutathione was thereby

Table 2. *Desulphurization of L-cystine by liver homogenates*

Media contained 0.067M-phosphate, pH 7.4 (1.5 ml.); liver homogenate (0.5 ml.) (see Methods section); 3 mM-NAD (0.1 ml.); 2 mM-pyridoxal phosphate (0.1 ml.); ethanol (0.1 ml.); solid cystine (5 mg.); added to Warburg flasks. The trapping and estimation of H<sub>2</sub>S were carried out as for the assay of cysteine desulphhydrase (see Methods section). Anaerobic incubation was at 37° for 4 hr., with constant shaking.

Case	Activity ( $\mu$ moles of H <sub>2</sub> S/g. dry wt. of liver homogenate/hr.)		
	Complete system	Ethanol omitted	Cystine omitted
Cystinosis 2	7.9	0	0.1
Cystinosis 3	11.8	2.2	0.3
Normal 1	7.4	0	0
Normal 2	8.2	0	0

Table 3. *Glutathione-reductase activity of liver*

Microcuvettes of 1 cm. light path contained: M-phosphate buffer, pH 7.6 (0.05 ml.); mM-NADPH<sub>2</sub> (0.05 ml.); 1% (w/v) serum albumin (0.05 ml.); extract of acetone-dried liver powder (0.05 ml.) (see Methods section); water (0.2 ml.); 30 mM-GSSG (0.05 ml.) or water. Extinctions at 340 m $\mu$  were measured at 30 sec. intervals for 5 min. at room temperature (22°).

Case	Period of tissue storage	Activity ( $-\Delta E_{340}$ /mg. of protein/min.)
Cystinosis 1	14 months	0.106
Cystinosis 2	9 months	0.121
Cystinosis 3	3 weeks	0.110
Normal 1	11 months	0.105
Normal 2	6 months	0.088

Table 4. *Reduction of cystine in the presence of reduced glutathione*

A. For experimental details of the overall reaction (enzymic + spontaneous), see Methods section. B. Spontaneous reaction; the medium contained: 0.1 M-tris buffer, pH 7.6 (1 ml.); 0.5 M-glucose 6-phosphate (0.2 ml.); 0.1 M-MgCl<sub>2</sub> (0.2 ml.); glucose 6-phosphate dehydrogenase (0.2 ml.  $\equiv$  10 units); 5 mM-NADP (0.1 ml.); 10 mM-GSH (0.2 ml.); glutathione reductase (0.05 ml.  $\equiv$  1000 units); solid cystine (5 mg.); in a final volume of 5 ml., contained in Warburg flasks. The experiment was otherwise performed as for (A). Results are expressed as changes occurring in 2 hr. at 37°.

A Case	Period of storage of liver	Difference in 5 mM-I <sub>2</sub> required (ml./ml. of reaction medium)					Complete system		
		Complete system	Cystine omitted	Glucose 6-phosphate omitted	GSH omitted	Glutathione reductase omitted	H <sub>2</sub> S* liberated ( $\mu$ moles/ml.)	Thiol groups formed Total ( $\mu$ moles/ml.)	Corrected† ( $\mu$ moles/mg. dry wt. of liver homogenate/ml.)
Cystinosis 2	6 months	+1.01	0	+0.02	+0.03	—	0.18	5.20	0.66
Cystinosis 3	5 weeks	+0.82	+0.03	-0.08	+0.03	—	0.22	4.37	0.63
Normal 1	11 months	+0.31	-0.03	+0.04	+0.01	—	0.27	1.85	0.03
Normal 2	3 months	+0.86	+0.02	+0.03	+0.02	—	0.31	4.60	0.61
B Spontaneous reaction	—	+0.34	+0.01	+0.02	0	0	0	1.70‡	—

\* No H<sub>2</sub>S was liberated from incomplete media.

† Calculated after subtraction of mean value for thiol groups formed in the spontaneous reaction.

‡ Range 1.43-1.95 (mean 1.71, from four estimations).

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