Biochem. J. (1969) **113**, 443
Printed in Great Britain

Distribution of Glutathione–Cystine Transhydrogenase Activity in Subcellular Fractions of Rat Intestinal Mucosa

By Beatrice States and Stanton Segal
Division of Biochemical Development and Molecular Diseases, Children's Hospital of Philadelphia and Department of Pediatrics, Medical School of the University of Pennsylvania, Philadelphia, Pa. 19146, *U.S.A.*

(*Received* 28 *March* 1969)

Two human metabolic disorders involving abnormal handling of cystine by cells are known: cystinuria, in which there is a renal loss of cystine (Dent, Senior & Walshe, 1954), and cystinosis, in which there is an abnormal accumulation of cystine (Seegmiller, Friedmann, Harrison, Wong & Schneider, 1968) although normally the intracellular form of the amino acid is mainly the reduced compound cysteine (Crawhall & Segal, 1967; States & Segal, 1968). Recent observations have caused concern about the cellular localization of the cystine-reductive process involved. First, in human intestinal mucosa from cystinuric patients there is defective membrane transport of cystine but normal transport of cysteine (Rosenberg, Crawhall & Segal, 1967), which raises the question whether there is reduction of cystine in the intestinal brush border as a concomitant of cystine transport. Secondly, cystine accumulated in cells of cystinotic patients is associated with cell fractions containing acid phosphatase (Schneider, Bradley & Seegmiller, 1967), and crystals have been shown to be enclosed by membranes staining positively for this phosphatase (Patrick & Lake, 1968), thus suggesting lysosomal cystine storage. The latter findings raise the possibility that in cystinosis there may be a defect in lysosomal reduction of cystine.

Although Wendell (1968) reported reduction of cystine by a supernatant fraction from rat gut mucosa containing glutathione–cystine transhydrogenase activity, and Nagai & Black (1968) isolated and partially purified a soluble enzyme with similar activity from yeast, the localization of glutathione–cystine transhydrogenase within the cell has not been investigated. We have therefore undertaken such an investigation in subcellular fractions of normal rat intestinal mucosa.

The small intestine from male Sprague–Dawley rats weighing 250–350g., killed by decapitation, was removed from the ligament of Treitz to the caecum, and placed in cold 0·15M-KCl. The gut was washed with cold 0·15M-KCl, everted (Wilson, 1962) and lightly scraped in the cold with a glass slide. Each gram of mucosa was added to 9ml. of cold 0·25M-D-mannitol, homogenized in a cold Corning–TenBroeck homogenizer, pulled through a 20-gauge needle into a 30ml. syringe and ejected slowly into a cold centrifuge tube. Fractionation was carried out in the cold in either a Serval SS-34 or a Spinco L-50 centrifuge by a modification of the method of Schneider & Hogeboom (1950), as follows. The homogenate was centrifuged for 10min. at 1500*g*. The 1500*g* supernatant was centrifuged for 20min. at 10000*g*. The sediment contained heavy mitochondria (fraction M_1). The 10000*g* supernatant remaining was centrifuged for 10min. at 33000*g*, resulting in sedimentation of the light mitochondria and lysosomes (fraction M_2). The 33000*g* supernatant was centrifuged for 1hr. at 105000*g*, leaving sedimented microsomes and supernatant cell sap. Two ml. of supernatant from each fractionation was withdrawn and tested directly for glutathione–cystine transhydrogenase activity. The precipitated fractions were suspended twice in 5ml. of 0·25M-D-mannitol and centrifuged twice for 10min. at 33000*g* before suspension in 2ml. of 0·1M-potassium phosphate buffer, pH6·5. The suspensions of fraction M_1 and microsomal fractions were sonicated in the cold for 30sec., and the M_2 fraction was sonicated for 90sec. The disrupted cellular materials were removed by centrifugation at 4° for 10min. at 33000*g* and the resulting supernatants tested for enzymic activity.

Brush borders, isolated by the method of Forstner, Sabesin & Isselbacher (1968), were suspended in 2ml. of 0·1M-potassium phosphate buffer, pH6·5, frozen and thawed ten times and centrifuged at 4° for 10min. at 33000*g*. Supernatant and precipitated material, after suspension in 2ml. of 0·1M-potassium phosphate buffer, pH6·5, were assayed for the enzymic activity.

To prepare a lysosome-rich fraction, M_2 fractions were resuspended in 5ml. of 0·25M-D-mannitol and centrifuged at 4° for 10min. at 10000*g*; the precipitate was discarded and the supernatant was centrifuged again for 10min. at 33000*g*. The supernatant was discarded and the precipitate was resuspended in 3ml. of 0·7 molal sucrose. Six sucrose-density-gradient tubes were prepared at least 20hr. before use and consisted initially of the following molalities: 1ml. of 0·264; 1ml. of 1·342; 1ml. of 2·387; and 2ml. of 3·420. Then 0·5ml. of the suspension of fraction M_2 was layered carefully on each tube. The tubes were centrifuged in the cold

Table 1. *Glutathione–cystine transhydrogenase activity in subcellular fractions*

The assay consisted of incubating 0·1 ml. of tissue extract at 25° for 5 min. in 0·1 ml. of 0·1 M-potassium phosphate buffer, pH 6·5, containing 0·01 ml. of 50 mM-EDTA, 0·01 ml. of 1·2 mM-NADPH, 0·01 ml. of 2·09 mM-cystine, 0·003 ml. of 2 mM-cystine containing approx. 0·2 μc of ^{35}S, and 0·01 ml. of 6·04 mM-GSH. Controls without tissue and with boiled tissue were tested for non-enzymic reduction of cystine. [This is a modification of the method of Wendell (1968).] The reaction was stopped by adding 0·3 ml. of 2 mM-*N*-ethylmaleimide (NEM) prepared in 0·1 M-potassium phosphate buffer, pH 6·5, and processed for t.l.c. to separate the glutathione–*N*-ethylmaleimide adduct, cysteine–*N*-ethylmaleimide adduct [i.e. 2-(L-2-amino-2-carboxyethylthio)-*N*-ethylsuccinimide] and cystine as described previously (States & Segal, 1969: solvent B4). Sections of the chromatogram were placed in a counting vial containing 10 ml. of phosphor scintillator and radioactivity was determined by counting in a Packard scintillation counter. Areas comparable with glutathione–*N*-ethylmaleimide adduct were found to contain less than 1% of the total ^{35}S radioactivity. Reduction of cystine to cysteine was based on the percentage of the total radioactivity recovered in cysteine–*N*-ethylmaleimide adduct × nmoles of original substrate.

Fraction	Total volume (ml.)	Protein (mg./ml.)	Cystine reduced (nmoles/min./mg. of protein)
Supernatant			
1500*g*	142	6·6	0·66
10000*g*	140	8·3	0·79
33000*g*	140	7·8	0·75
105000*g*	140	5·4	1·10
Fraction M_1*	2	4·0	0·02
Fraction M_2*	2	3·3	0·21
Microsomes*	2	0·7	0·01
Light mitochondria†	1	12·8	0·00
Lysosome-rich*	1	1·55	1·75
	2	1·40	1·18‡
Brush borders*			
Particulate	2	10·0	0·00
33000*g*	2	0·03	0·00‡

* See the text for description.

† Light-mitochondrial fraction after separation from lysosomes on a sucrose density gradient.

‡ 0·2 ml. of extract replaced 0·1 ml. of extract + 0·1 ml. of 0·1 M-phosphate buffer, pH 6·5, in the assay.

for 1 hr. at 105000*g*. The precipitated lysosome-rich fractions were pooled and suspended in 1 or 2 ml. of 0·1 M-potassium phosphate buffer, pH 6·5, frozen and thawed ten times and centrifuged at 4° for 10 min. at 33000*g*. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Table 1 shows the distribution of glutathione–cystine transhydrogenase activity in cellular constituents and supernatants of rat intestinal mucosa. Three observations may be made based on the results presented in Table 1. First, there appears to be no glutathione–cystine transhydrogenase activity in the brush borders of the intestine. Therefore the previous assumption (States & Segal, 1968) that transport of cystine by the intestine *in vitro* is not limited by cystine reduction to cysteine within the brush border before or during transport is substantiated. Secondly, the cell sap, i.e. the 105000*g* supernatant, appears to contain the major glutathione–cystine transhydrogenase activity. Thirdly, the finding of cystine-reducing activity in the lysosome-rich fraction may have a significant bearing on the deposition of cystine in lysosomes in cystinosis (Patrick & Lake, 1968). Recently a number of reports have appeared in the literature (Beck, Mahadevan, Brightwell, Dillard & Tappel, 1968; Coffey & de Duve, 1968; Aronson & de Duve, 1968; Fowler & de Duve, 1969; Tudball & Davidson, 1969; Alpers, 1969) on the isolation and partial purification of lysosomal enzymes, and it is conceivable that a future thorough investigation of the optimum conditions of lysosomal reduction of cystine may lead to a better understanding of human cystinosis.

This work was supported by grants from John A. Hartford Foundation and National Institutes of Health (Grant AM 10894).

Alpers, D. H. (1969). *J. biol. Chem.* **244**, 1238.
Aronson, N. N., jun. & de Duve, C. (1968). *J. biol. Chem.* **243**, 4564.
Beck, C., Mahadevan, S., Brightwell, R., Dillard, C. J. & Tappel, A. L. (1968). *Arch. Biochem. Biophys.* **128**, 369.
Coffey, J. W. & de Duve, C. (1968). *J. biol. Chem.* **243**, 3255.
Crawhall, J. C. & Segal, S. (1967). *Biochem. J.* **105**, 894.
Dent, C. E., Senior, B. & Walshe, J. M. (1954). *J. clin. Invest.* **33**, 1216.
Forstner, G. G., Sabesin, S. M. & Isselbacher, K. J. (1968). *Biochem. J.* **106**, 381.
Fowler, S. & de Duve, C. (1969). *J. biol. Chem.* **244**, 471.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Nagai, S. & Black, S. (1968). *J. biol. Chem.* **243**, 1942.
Patrick, A. D. & Lake, B. D. (1968). *J. clin. Path.* **21**, 571.
Rosenberg, L. E., Crawhall, J. C. & Segal, S. (1967). *J. clin. Invest.* **46**, 30.
Schneider, J. A., Bradley, K. & Seegmiller, J. E. (1967). *Science*, **157**, 1321.
Schneider, W. C. & Hogeboom, G. H. (1950). *J. biol. Chem.* **183**, 123.
Seegmiller, J. E., Friedmann, T., Harrison, H. E., Wong, V. & Schneider, J. A. (1968). *Ann. intern. Med.* **68**, 883.
States, B. & Segal, S. (1968). *Biochim. biophys. Acta*, **163**, 154.
States, B. & Segal, S. (1969). *Analyt. Biochem.* **27**, 323.
Tudball, N. & Davidson, E. A. (1969). *Biochim. biophys. Acta*, **171**, 113.
Wendell, P. L. (1968). *Biochim. biophys. Acta*, **159**, 179.
Wilson, T. H. (1962). *Intestinal Absorption*, p. 32. Philadelphia: W. B. Saunders Co.