Transport and Intracellular Fate of Cysteine-³⁵S in Leukocytes from Normal Subjects and Patients with Cystinosis

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Extract

The transport and intracellular fate of cysteine-³⁵S was studied in leukocytes from nine children with cystinosis and nine control subjects of similar age in an attempt to explain the presence of 80 times the normal quantity of free cystine in cystinotic leukocytes. Two differences were observed: the cystinotic cells took up nearly twice as much cysteine-³⁵S as did control cells. Subsequently, almost 40 percent of the ³⁵S was found as the oxidized form, cystine-³⁵S in cystinotic leukocytes, compared with only 2 percent in control leukocytes. Comparison of intracellular and extracellular concentrations of cysteine-³⁵S at steady state showed no differences between normal and cystinotic leukocytes. Both cell types incorporated 30 percent of the intracellular ³⁵S into glutathione and showed the same time course of ³⁵S efflux.

Speculation

The increased uptake of cysteine-³⁵S shown by cystinotic leukocytes could be taken as valid conventional evidence for a primary defect in the transport of cysteine in cystinosis. The prompt appearance of the extra ³⁵S transported as intracellular cystine-³⁵S suggests, however, that this rapid conversion may be the primary defect and that the increased uptake of cysteine-³⁵S is but a compensatory mechanism for maintaining the physiological concentration of intracellular cysteine.

Introduction

The basic defect in metabolism leading to the formation of cystine crystals in the rare inherited disease, cystinosis, is not known [2, 27, 30]. The cystine is thought to cause both the renal tubular defects of the Fanconi syndrome which appear during the first year of life, and the progressive renal glomerular damage which leads to death of affected children [12, 20]. Careful studies of postmortem tissue from these patients have revealed normal activity of all enzymes tested, which included most of those known to be necessary for cys-

tine metabolism [19]. We have recently found eighty times as much free-cystine in cystinotic as compared with normal leukocytes [23]. This fact, coupled with the normal plasma concentration of cystine found in these children [4], suggested a defect in the transport of cyst(e)ine (cystine and/or cysteine) in cystinotic cells, analogous to the apparent defect in cystine transport in cells of the renal tubules in the otherwise unrelated disease, cystinuria [11, 17, 26, 29]. The lack of suitable tissue from infants and small children with this disease has been a major obstacle to definitive studies of transport. Leukocytes provide a readily

available tissue for study, although ROSENBERG [22] found poor uptake of cystine by normal leukocytes. We have confirmed this observation in both normal and cystinotic leukocytes; however, both types of leukocytes showed a substantial uptake of the reduced form, cysteine. A new technique, the use of thiolated sephadex [10, 15] to maintain extracellular cysteine in the reduced form, permitted the comparison of cysteine- ^{35}S transport in normal and cystinotic leukocytes.

Methods

Subjects Studied

Nine infants and children with cystinosis, ranging in age from 5 months to 9 years, were studied. Nine children of similar ages served as controls. Two of the control subjects had been admitted to the Clinical Center for elective cardiac catheterization because of acyanotic congenital heart disease. One had idiopathic hypoglycemia; the other six children were normal. All were having blood taken for other purposes and 10 to 20 ml of blood was obtained for these studies. Normal adults also served as donors.

Leukocyte Isolation

Ten to forty ml of blood, usually obtained after an overnight fast, was mixed immediately with 20 times its volume of normal saline to prevent clotting [3]. The mixture was centrifuged at $700 \times g$ for 20 minutes in 250 ml glass bottles to collect all cells. The supernatant was discarded and erythrocytes disrupted by exposure to 90 ml of 0.2 % NaCl for 20 seconds, followed by 90 ml of 1.6 % NaCl. After centrifugation, the supernatant hemolysate was removed by aspiration and discarded, taking care not to disturb the leukocytes. The remaining erythrocytes were hemolyzed by the addition to the cellular suspension of 6 ml of water and 15 seconds later of 2 ml of 3.6 % NaCl. The cells were then centrifuged in 15 ml cellulose nitrate tubes for 10 minutes at $120 \times g$ and again washed with normal saline. Care was taken to remove any erythrocyte ghosts which remained above the leukocyte pellet. All solutions were kept at 4° and all procedures were done at this temperature. All glassware was siliconized. This method proved especially suited for isolating leukocytes from small volumes of blood.

Leukocyte Incubations

Twenty to forty million leukocytes, suspended in 2.5 ml of Krebs-Ringer bicarbonate buffer, were incubated in 30 ml polyethylene bottles in an atmosphere of 95 % oxygen and 5 % carbon dioxide at 37° , which maintained a pH of 7.4 throughout all incubations.

The usual concentration of cysteine- ^{35}S was 0.03 mM and of cystine- ^{35}S , 0.02 mM. A Dubnoff shaker was used to agitate the cell suspension at a rate of 90 oscillations per minute. Cysteine- ^{35}S was prepared from cystine- ^{35}S by reduction with an electrolytic desalter [31] for six minutes (initial current, 0.6 A) [5] prior to the incubation, and was kept reduced by the use of approximately 25 mg (wet weight) of thiolated sephadex [10, 15]. No clumping of leukocytes was observed after an incubation period as long as 90 minutes. Other workers [6] have used dithiothreitol to keep cysteine reduced, but under our conditions this caused clumping of leukocytes. Because the volume of the cellular material was small compared with the volume of the incubation media, the extracellular amino acid concentration remained essentially unchanged during the incubations.

The incubations were terminated by pipetting the entire mixture into 15 ml cellulose nitrate tubes in an ice bath. The suspensions were immediately centrifuged at $120 \times g$ for 10 minutes at 4° . Virtually no transport of amino acids occurs at this temperature [22]. The supernatant was saved and the leukocytes and thiolated sephadex suspended in 2 ml of normal saline (4°) and then immediately centrifuged in pre-cooled Bauer-Schenck tubes [32] at $2200 \times g$ for 10 minutes at 4° . The volumes of both the sephadex, which layered at the bottom, and the leukocytes were recorded.

In order to prevent oxidation of cysteine following cell lysis, 0.5 ml of 20 mM N-ethylmaleimide (NEM) in 0.01 M potassium phosphate buffer, pH 7.4, was added [28] to the cells and sephadex; the mixture was transferred to a 2 ml conical centrifuge tube with a long-tipped Pasteur pipette. The leukocytes were then lysed with a Branson Sonifier [33], using a special 'blood microtip' at a current of 2 A for 30 seconds. After a lapse of one minute for the reaction of NEM with cysteine to form 2-(L-2-amino-2-carboxyethylthio)-N-ethyl succinimide (cysteine-NEM), 0.5 ml of 3 % sulfosalicylic acid was added and the precipitated protein removed by centrifugation. The supernatant was used for radioisotope analysis and will be referred to as the 'cellular extract'.

For the study of efflux, leukocytes were incubated with cysteine- ^{35}S as described above, but thiolated sephadex was not used since it was not necessary to keep the cysteine- ^{35}S completely reduced. After incubation (60 minutes), the leukocytes were washed twice with 5 ml Krebs-Ringer bicarbonate buffer and then placed in 2 ml of this buffer at 37° in 30 ml polyethylene bottles. The bottles were incubated in a Gyrotory shaker [34] at 60 oscillations per minute, at 37° under 95 % oxygen and 5 % carbon dioxide. Every four minutes the bottle was quickly removed and centrifuged

in a specially designed holder for 15 seconds, reaching a maximum of $500 \times g$. Fifty μ l were removed from the incubation media for radioactive assay and the cells were then resuspended and the incubation continued.

Amino Acid Analysis

Amino acids were separated on 3 mm Whatman paper by electrophoresis in 6.8 % formic acid (vol/vol) at 1300 V for $1\frac{1}{2}$ hours at 0°, using a 'Pherograph' apparatus [35]. Amino acids were located by staining with ninhydrin [1]. For ion-exchange chromatography, a Beckman Model 120 B automatic amino acid analyzer was employed using Crawhall's gradient elution technique, which is especially suitable for the separation of sulfur-containing amino acids [8]. To increase sensitivity, the colorimeter cuvettes and recorder were assembled as described by HUBBARD and KREMAN [14].

Radioisotope Analysis

Radioactivity was measured by mixing 0.050 ml of solution with 1 ml absolute alcohol and 10 ml of a toluene-based counting solution [36]. A Packard Model 2002 Tri-Carb scintillation counter was used (room temperature) at a counting efficiency of 72 % for ³⁵S with a background of 15 cpm. Following high voltage electrophoresis on paper, radioactivity was located either by radioautography, using Kodak non-screen x-ray film, or by cutting the paper into 2 cm segments and counting each in 5 ml of the scintillation solution. Alternatively, effluent from the ion exchange column was passed through anthracene crystals packed in a 2 ml flow cell (Packard Model 3043), in the above-mentioned scintillation counter, prior to mixing with ninhydrin. At a flow rate of 50 ml per hour, a counting efficiency of 55 % for ³⁵S with a background of 12 cpm was obtained.

Calculation of the Distribution Ratio

The distribution ratio was calculated as described by ROSENBERG [22] and represents the ratio of the concentrations of intracellular to extracellular radioactivity. A correction was required, however, for the small amount of cysteine-³⁵S which remained attached to the thiolated sephadex. A good correlation was found between the volume of sephadex as measured in the Bauer-Schenck tube and its contribution to the radioactivity in the cellular extract after standard incubations.

Calculation of Efflux Rate

At the completion of each experiment, the radioactivity remaining within cells and in the incubation media was determined as described above. These data, together with the data from the samples removed,

allowed the computation of the total intracellular radioactivity at the start of the incubation. Knowing the concentration of radioactivity (cpm per 0.050 ml as described above) and the volume of incubation media at the end of each four-minute period, we were able to calculate the amount of radioactivity which had left the cells during each period. This was expressed as a percent of the remaining radioactivity and plotted logarithmically as a function of time. The percent loss per minute was determined from the slope of this line.

Materials

Cystine-³⁵S (S.A. 88 mC/mmole) was obtained commercially [37]. A fresh solution was made weekly and 98 % of the radioactivity migrated identically with authentic cystine on high voltage paper electrophoresis and corresponded to the only ninhydrin-positive spot. When the flow cell was used in conjunction with the amino acid analyzer, approximately 2 % of the ³⁵S was found to be eluted close to the solvent front in the same region as cysteic acid, cysteine sulfinic acid or β -mercaptopyruvate, and approximately 98 % of the ³⁵S was eluted at the cystine region. After a storage period of two weeks, more than 5 % of the ³⁵S migrated at the solvent front. This analytical system showed that only 75 % (by weight) of this material eluted as cystine, and the remainder did not react with ninhydrin and did not contain ³⁵S (except for the 2 % mentioned above). Thus, the specific activity was higher than that claimed by the manufacturer.

Thiolated sephadex was prepared from sephadex [38] as described by ELDJARN and JELLUM [10, 15]. Inulin-carboxyl-¹⁴C (S.A. 3 mC/g with an average molecular weight of 5000–5500) [39] was used for determining extracellular volume as described by ROSENBERG [22]. Arginine-¹⁴C (U.L., S.A. 220 mC/mmole) was also obtained commercially [39], as was glucose-1-¹⁴C (S.A. 1.72 mC/mmole) [40].

Results

Properties of the Isolated Leukocytes

Efficiency of recovery of leukocytes from peripheral blood was approximately 50 %, and was somewhat greater for polymorphonuclear leukocytes than for lymphocytes. Smears of these preparations stained with either Giemsa or Wright's stain revealed very few erythrocytes and only rare platelets. The leukocytes appeared normal by phase microscopy and fewer than 5 % of the cells were permeable to 0.025 % trypan blue, a dye which stains only nonviable cells [18]. When the leukocytes were incubated with glucose-1-¹⁴C, carbon dioxide-¹⁴C was evolved at a constant rate for at least 60 minutes.

Studies of total tissue water agreed closely with the findings of ROSENBERG [22] and a value of 85 % was used in all calculations. The inulin space was 11.4 ± 1.6 % of the packed cell volume and differs from a previous report in which a value of 42.3 ± 6.0 % was noted [22]. This difference may reflect the higher speeds at which the cells were centrifuged to form the final leukocyte pellets or the higher molecular weight of the inulin preparations used.

Use of Thiolated Sephadex

This material was very effective in maintaining extracellular cysteine in the reduced state [10, 15]. When 4 ml of 0.33 mM cysteine (11 times the concentration used for the transport studies) was incubated for two hours in 95 % oxygen and 5 % carbon dioxide in Krebs-Ringer bicarbonate buffer at 37°, pH 7.4, the cysteine was kept completely reduced by 25 mg but not by 5 mg (wet weight) of thiolated sephadex. The presence of this material had no effect on the uptake of arginine- ^{14}C by normal leukocytes. The sephadex was stored in suspension and blotted dry before use.

A small amount of cysteine- ^{35}S remained bound to the thiolated sephadex. To correct for this, cysteine- ^{35}S was incubated with varying amounts of sephadex in the absence of leukocytes, and the correction curve obtained related the volume of sephadex to the amount of cysteine- ^{35}S bound to sephadex.

In a typical leukocyte incubation, about 0.03 % of the total cysteine- ^{35}S remained bound to the thiolated sephadex, and the sephadex pellet contained about 10 percent as much radioactivity as did the leukocyte pellet. Studies with NEM demonstrated that only the reduced form, cysteine- ^{35}S , was released into the 'cellular extract' from thiolated sephadex.

Uptake of Cysteine

The distribution ratio of the concentrations of intracellular to extracellular radioactivity was very low after incubation in 0.02 mM cysteine- ^{35}S . The ratio was less than 2 after an incubation period of up to 2 hours for both normal and cystinotic leukocytes. The distribution ratio of ^{35}S was much greater, however, after incubation in 0.03 mM cysteine- ^{35}S , often reaching 20 by 90 minutes (fig. 1). The relatively small volume of blood available precluded kinetic studies. Instead, distribution ratios were compared at a standard cysteine- ^{35}S concentration of 0.03 mM, which is within the range of cysteine concentration of 0.006 to 0.035 mM reported for normal and cystinotic plasma [4]. Ninety minutes was selected as the standard incubation time in order to enhance any differences between normal and cystinotic leukocytes.

The distribution ratio of ^{35}S following incubation in cysteine- ^{35}S was significantly higher in leukocytes

from 9 children with cystinosis than in those from 9 control children of similar age. The value was 34 ± 7.7 (mean ± 1 SD) for the cystinotic leukocytes as compared with a value of 21 ± 8.3 in leukocytes from the control group ($p < 0.01$).

Fate of Intracellular Cysteine

The differences in distribution ratios would be most meaningful if the intracellular ^{35}S in both normal and cystinotic cells remained as cysteine- ^{35}S , the form present in the incubation media, and the predominant intracellular form of this amino acid in all tissues previously studied [7, 21]. Separation of intracellular components by high voltage electrophoresis on paper,

Fig. 1. The time course of arginine- ^{14}C and cysteine- ^{35}S uptake by normal leukocytes. The incubation media contained 0.065 mM arginine- ^{14}C or 0.03 mM cysteine- ^{35}S .

Fig. 3. The combined analysis of cellular extracts by flow counter and amino acid analyzer. A mixture of 15 μmoles cysteine-NEM, 25 μmoles glutathione-NEM and 5 μmoles of the other amino acids seen was added as carrier to cell extracts containing 4 to 5 μC of ^{35}S . The two isomers of cysteine-NEM elute separately in this system [8]. Significant radioactivity is eluted with cysteine in cystinotic and not in normal extracts. Glutathione-NEM exaggerates the leading edge of the aspartic acid peak and was associated with significant radioactivity in both cases. Reduced glutathione (GSH) is eluted about 10 minutes prior to glutathione-NEM in this system. (The cellular extracts are not the ones seen in figure 2.)

Fig. 2. Separation of cysteine- ^{35}S and its metabolites from normal and cystinotic leukocytes by high voltage electrophoresis on paper. Cellular extracts were electrophoresed in the center channels and standard compounds in the outer channels. A composite of ninhydrin stained standards and radioautographed extracts is shown. The radioactive component migrating just behind cysteine-NEM has been shown to be glutathione-NEM. About 2 μCi was present in each extract and the radioautograms were exposed for 30 days.

followed by radioautography, revealed two highly radioactive components in normal cells, corresponding to cysteine-NEM and glutathione-NEM. Cystinotic cells also showed these components as well as a third component corresponding to cystine (fig. 2). The identity of glutathione-NEM was confirmed by the use of the flow counter in conjunction with the amino acid analyzer. As seen in figure 3, significant peaks of radioactivity were eluted with glutathione-NEM in both cellular extracts. When the leukocytes were lysed without NEM, the glutathione-NEM peak was replaced by two separate peaks which eluted respectively with the reduced and oxidized forms of glutathione.

After incubation (90 minutes), the intracellular metabolites of cysteine- ^{35}S were determined by ion exchange chromatography and the flow counter. The results are summarized in table I. Corrections were made for the cysteine- ^{35}S released from thiolated sephadex. Approximately 30 percent of the ^{35}S was present as glutathione- ^{35}S -NEM in both cystinotic and normal leukocytes, but the percentage present as cysteine- ^{35}S -NEM in normal cells was twice that in cystinotic cells. Cystinotic leukocytes contained nearly 40 percent of the ^{35}S as cystine- ^{35}S , compared with about 2 percent in normal cells. Essentially the same distribution of ^{35}S was found after only 30 minutes of incubation. When leukocytes from a child with cystinosis were incubated with cysteine- ^{35}S for only three minutes, 16 percent of the intracellular ^{35}S was found as glutathione- ^{35}S , 44 percent as cysteine- ^{35}S -NEM and 34 percent as cystine- ^{35}S .

Table I. The identification of ^{35}S in leukocytes from cystinotic and control children after incubation for 90 minutes in 0.03 mM cysteine- ^{35}S

	Percent total ^{35}S ¹	
	Control children	Cystinosis
Cysteic acid, cysteine sulfinic acid, etc. ²	7.6 ± 1.6^3	3.2 ± 1.8
Glutathione-NEM	30.2 ± 13.5	27.1 ± 9.5
Cysteine-NEM	60.2 ± 13.4	30.4 ± 9.4
Cystine	2.3 ± 1.3	39.3 ± 4.9

¹ Determined by the combined use of a flow counter and amino acid analyzer. Correction has been made for the cysteine- ^{35}S which remains attached to the thiolated sephadex.

² Compounds which elute very near the solvent front and are not separated from each other by the method used.

³ Mean $\pm$ 1 SD.

Using these data, the distribution ratio of cysteine- ^{35}S at 90 minutes was 10 ± 3.2 in cystinotic cells compared with 13 ± 6.9 in normal cells ($p > 0.2$).

Efflux of ^{35}S

The efflux of ^{35}S from cells after loading the cells by incubation with cysteine- ^{35}S was 1.0 ± 0.38 percent loss per minute in leukocytes from 6 patients with cystinosis as compared with 0.92 ± 0.38 percent loss per minute in leukocytes from 5 control subjects (fig. 4). At the completion of the efflux experiment (20 minutes) a portion of the suspending media was immediately treated with NEM and radioactive components were identified by ion-exchange chromatography and flow counting. In both groups, more than 50 % of the ^{35}S was identified as cysteine- ^{35}S -NEM and most of the remainder as cystine- ^{35}S . Less than 5 % of ^{35}S was eluted at the solvent front or with glutathione-NEM. If we assume that ^{35}S leaves the leukocytes as cysteine- ^{35}S , and consider the efflux as a percent of the cysteine- ^{35}S loss, the cystinotic leukocytes showed an efflux of cysteine twice that of normal cells because they contained half as much of their intracellular ^{35}S as cysteine- ^{35}S .

Intracellular Specific Activity

For determination of intracellular specific activity, the incubations were performed in the usual manner, but the entire cellular extract was assayed by combined ion-exchange chromatography and flow counting. This was necessary in order to measure accurately the molar quantities of cysteine- ^{35}S -NEM and cystine- ^{35}S . The findings are summarized in table II. After incubation of leukocytes (90 minutes) from both control subjects and cystinotic patients, the specific activity of intracellular cysteine- ^{35}S was considerably decreased, compared with that of extracellular cysteine- ^{35}S . The specific activity of cystine- ^{35}S was lower than that of

Fig. 4. Efflux of cyst(e)ine- ^{35}S from normal and control leukocytes. The efflux rate was obtained from the slope of the line between 4 and 20 minutes.

Table II. Intracellular distribution and specific activity (S.A.) of free-cyst(e)ine-³⁵S in leukocytes when incubated in 0.03 mM cysteine-³⁵S¹

	0 minutes		90 minutes					
	Cys-NEM	Cystine	Cysteine- ³⁵ S-NEM		Cystine- ³⁵ S		Total cyst(e)ine- ³⁵ S	Transported cysteine- ³⁵ S ³
	(A)		(B)		(C)		(D = B + C)	(E)
	mμmole ²		mμmole ²	S.A.	mμmole ²	S.A.	mμmole ²	mμmole ²
Normal								
10-year-old male			1.45	6.0	0.07	5.0	1.52	0.43
24-year-old female	< 0.08 ⁴	0.07	0.47	5.3	0.06	3.5	0.53	0.13
Cystinotic								
8-year-old male	< 0.25 ⁴	7.5	2.7	5.8	6.3	2.6	9.0	1.5
4-year-old male	< 0.29 ⁴	6.8 ⁵	2.8	3.8	6.1	2.5	8.9	1.2

¹ The specific activity of the extracellular cysteine-³⁵S was 21 mμC/mμmole ½cystine at the start of incubation.

² mμmole ½ cystine/mg protein; protein was determined colorimetrically [16].

³ The value in this column is calculated from the mμC of intracellular cyst(e)ine-³⁵S following the incubation, and the specific activity of the extracellular cysteine-³⁵S at the start of the incubation. It represents the net labeled cysteine which has entered the cell during incubation.

⁴ Not detectable. The value shown is the least amount that could have been detected.

⁵ Leukocytes prepared at a different time than those used for the transport study.

cysteine-³⁵S in all cases. The total mμmole ½ cystine per mg of protein at zero time (table II, column A) plus that which entered the cell during the incubation (column E) matched closely the value measured at 90 minutes (column D) in the leukocytes from the children with cystinosis. In leukocytes from both these patients, the amount of the oxidized form, cystine, decreased slightly during the incubation.

Discussion

Following incubation with cysteine-³⁵S, a significantly higher concentration of ³⁵S was found in leukocytes from cystinotic patients than in leukocytes from control subjects. In cystinuria, such a difference has been taken as evidence for an altered transport mechanism [11, 17, 26, 29]. The interpretation of such data in these studies, however, was complicated by the high initial content of cystine in the cystinotic cells. It was important to determine whether an increased uptake of cysteine led to the cystine accumulation, or whether the large cystine pool caused the increased uptake of cysteine by these cells. We attempted to control these studies by loading normal cells with cystine crystals prior to incubation with cysteine-³⁵S. When control leukocytes were allowed to ingest microcrystals of cystine, however, they immediately clumped together and were no longer suitable for transport studies.

Two possible mechanisms could explain why cystinotic cells, preloaded with cystine, would take up increased amounts of cysteine-³⁵S. There could be an exchange diffusion of cysteine moieties across the cell membrane. The phenomenon was described by HEINZ in Ehrlich tumor cells; they demonstrate an increased influx of glycine-¹⁴C when preloaded with glycine-¹²C [13]. Alternatively, the explanation of the increased cysteine-³⁵S uptake by cystinotic leukocytes could be related to the abnormally high percentage of ³⁵S which is found as cystine-³⁵S in these cells following incubation with cysteine-³⁵S. This might occur because of altered oxidation-reduction mechanisms or by a simple exchange of half cystine moieties between the two forms of this amino acid. The determinations of intracellular specific activity of cysteine-³⁵S and cystine-³⁵S were obtained to clarify this problem. Complete exchange of half cystines during the 90-minute incubation period would have resulted in nearly identical intracellular specific activities for these two compounds. In contrast, increased oxidation of transported cysteine-³⁵S would lead to an increased amount of intracellular cystine following the incubation.

As seen in table II, a complete exchange did not occur; the specific activity of the oxidized form, following incubation, was approximately one-half that of the reduced form in cystinotic cells. The molar quantity of cystine, however, actually decreased during the incubation in both experiments with cystinotic leukocytes.

Thus, there was no indication of an abnormality in oxidation-reduction mechanisms, and a disulfide exchange reaction remains the most likely explanation for the increased amount of ^{35}S present in cystine- ^{35}S in cystinotic leukocytes after incubation with cysteine- ^{35}S .

The decrease in specific activity of intracellular cysteine- ^{35}S may have resulted from dilution by a variety of processes. These include cysteine liberated by glutathione turnover, *de novo* synthesis of cysteine, disulfide exchange with either protein-bound cysteine [9] or dilution by the intracellular pool of free cyst(e)ine. Regardless of the cause of the decrease in specific activity, the experimental results mean that the true distribution ratio of cysteine is greater than the value simply determined by the ratio of intracellular to extracellular radioactivity in cysteine- ^{35}S . Since the decrease in intracellular specific activity was approximately the same in the types of leukocytes studied, a comparison between these groups remains valid. This may not be true, however, in other instances in which distribution ratios were compared on the basis of radioactivity alone [11, 17, 21, 29].

Another factor which made these studies difficult to interpret was the large amount of ^{35}S that was found as glutathione following the incubations. Glutathione- ^{35}S -NEM migrates so close to cysteine- ^{35}S -NEM in the electrophoretic system commonly used (fig. 2) that if it were formed in other tissues, it could easily be regarded as cysteine- ^{35}S -NEM in studies of cyst(e)ine- ^{35}S transport [21]. We have observed similar incorporation of cysteine- ^{35}S into glutathione- ^{35}S in cultured human skin fibroblasts [24]. Since a similar percentage of ^{35}S was found in glutathione- ^{35}S in both control and cystinotic cells, it did not affect the comparisons made in this study.

The efflux of ^{35}S from normal and cystinotic cells was quite similar, suggesting that there was no diminution of efflux to account for the cystine accumulation of cystinotic cells. The fact that over 50 percent of the ^{35}S in the extracellular medium was cysteine- ^{35}S , despite the known spontaneous oxidation of cysteine in this environment, suggests that the major efflux mechanism involves the reduced form. Since the magnitude of the efflux of the disulfide form cannot be determined, a direct comparison of the influx to efflux rate of cysteine- ^{35}S is not possible. Use of thiolated sephadex in efflux experiments would not have solved the problem but would have introduced further problems by binding varying quantities of cysteine- ^{35}S as the concentration of extracellular ^{35}S increased.

Intracellular ^{35}S is found as both glutathione- ^{35}S and cystine- ^{35}S after incubating cystinotic leukocytes with cysteine- ^{35}S for periods as short as three minutes. Therefore, kinetic studies of transport velocity at very early times would involve the same difficulties experi-

enced in these studies at conditions more nearly approaching a steady state. Because of these difficulties, a definitive elucidation of cyst(e)ine transport by cystinotic cells will probably require the preparation of isolated cell membranes.

Although the cause of the high cystine content of cystinotic cells remains unknown, we have recently reported that three-quarters of the intracellular cystine in cystinotic leukocytes is present in the 'granular' fraction after subcellular fractionation [23]. This finding has led us to believe that the defect in cystinosis may involve an abnormality of the intracellular compartmentalization of cystine, which could explain the intracellular accumulation of cystine in this disease in spite of a normal transport of cyst(e)ine across the cell membrane.

Summary

After incubating leukocytes in cysteine- ^{35}S , the distribution ratio of ^{35}S was 34 ± 7.7 in cystinotic leukocytes compared to 21.0 ± 8.3 in control leukocytes ($p < 0.01$); however, the distribution ratio of cysteine- ^{35}S was 10 ± 3.2 in cystinotic cells compared with 13.0 ± 6.9 in normal cells ($p > 0.2$).

Efflux of ^{35}S from cystinotic leukocytes was 1.0 ± 0.38 percent loss per minute compared to 0.92 ± 0.38 in normal cells.

Thirty percent of the transported cysteine- ^{35}S was incorporated into glutathione in both normal and cystinotic leukocytes.

39.3 ± 4.9 percent of the transported ^{35}S was found as cystine- ^{35}S intracellularly in cystinotic leukocytes compared with 2.3 ± 1.3 percent in normal leukocytes. An exchange of cystine moieties with the large pre-existing intracellular pool of cystine in the cystinotic leukocytes could account for the latter observation.

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