pH effects on cystine transport in lysosome-rich leucocyte granular fractions

William A. GAHL*† and Frank TIETZE‡

*The Interinstitute Genetics Program, National Institutes of Health, †Section on Biochemical Genetics, National Institute of Child Health and Human Development, National Institutes of Health, and ‡Section on Intermediary Metabolism, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20205, U.S.A.

(Received 1 March 1985; accepted 13 March 1985)

Inhibitors of lysosomal acidification (4,4'-di-isothiocyanostilbene-2,2'-disulphonate, *NN'*-dicyclohexylcarbodi-imide, carbonyl cyanide *m*-chlorophenylhydrazone, NH_4Cl and methylamine hydrochloride) did not alter cystine egress or counter-transport in polymorphonuclear-leucocyte lysosome-rich granular fractions at pH 7.0. Together, 2 mM- $\text{MgCl}_2/\text{MgATP}$ and 90 mM-KCl stimulated cystine egress 2-fold, but this effect also was not influenced by inhibitors of ATP-dependent lysosomal acidification. $\text{MgCl}_2/\text{MgATP}$ stimulated cystine transport at pH 5.5, but the effect also occurred with MgCl_2 , MgSO_4 or MnCl_2 alone, was prevented by chelation, and was not seen with NaATP; therefore, it was considered a bivalent-cation, not an ATP, effect. Proton-pump-mediated acidification of lysosomes does not appear to be required for cystine transport in normal polymorphonuclear-leucocyte granular fractions, as reported for lymphoblast lysosomes.

Cystine movement across normal lysosomal membranes exhibits saturation kinetics (Gahl *et al.*, 1982a) and *trans*-activation or counter-transport (Gahl *et al.*, 1983), both of which indicate a carrier-mediated process (Wilbrandt & Rosenberg, 1961). This facilitated cystine transport appears to be defective in cystinosis (Gahl *et al.*, 1982a,b; Jonas *et al.*, 1982), an autosomal, recessively inherited, lysosomal storage disease resulting in cystine accumulation within cellular lysosomes (Schneider & Schulman, 1982). Obligate heterozygotes for cystinosis display half the normal cystine-transporting capacity across the lysosomal membrane (Gahl *et al.*, 1982a, 1984). Characteristics of the normal lysosomal cystine-transport system include stereospecificity for the L-isomer of cystine, lack of competition by L-amino acids other than cystathionine, and temperature-dependence (Gahl *et al.*, 1983).

As in other transport systems, the lysosomal cystine carrier was examined for its behaviour in the presence of ATP. With lysosome-rich granular fractions from normal leucocytes, 1.25 mM- MgATP and - MgCl_2 stimulated cystine egress 2.5-fold in sucrose/Hepes buffer, pH 7.0, in the presence of 94 mM-KCl (Gahl *et al.*, 1982a). In

addition, several conditions that allowed K^+ free entry into lysosomes also stimulated cystine egress (Bashan *et al.*, 1984). Previously, Jonas *et al.* (1982), using lymphoblast granular fractions, reported that cystine egress was stimulated by 2 mM- ATP/MgCl_2 in the absence of KCl. The effect was ascribed to proton-pump-mediated lysosomal acidification (Jonas *et al.*, 1982, 1983). Using inhibitors of lysosomal acidification and different pH values, we examined cystine egress and counter-transport to determine if cystine transport was dependent on lysosomal acidification in normal polymorphonuclear-leucocyte granular fractions as well as in lymphoblasts.

Experimental

Materials

L-Cystine dimethyl ester, CCCP, 4-methylumbelliferyl 2-acetamido-2-deoxy- β -D-glucopyranoside, DIDS (disodium salt), MgATP (1.5 mol of Mg :1 mol of ATP), Na_2ATP , NaADP, NaGTP and methylamine hydrochloride were purchased from Sigma. NEM and DCCD were products of Pierce Chemical Co. L-[^3H]Cystine (2.7 or 3.2 Ci/mmol) was obtained from Amersham. Na_2EDTA was a product of Fisher Scientific Co., and NH_4Cl was purchased from Allied Chemical Co. CCCP was made fresh as a 5 mM stock solution in ethanol; DIDS and DCCD were made fresh as 10 mM stock solutions in dimethyl sulfoxide.

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonate; NEM, *N*-ethylmaleimide; DCCD, *NN'*-dicyclohexylcarbodi-imide.

Cystine egress

Human polymorphonuclear-rich leucocytes were loaded with cystine by incubation with 1 mM non-radioactive cystine dimethyl ester (Gahl *et al.*, 1982b). Granular fractions, containing over 4 nmol of half-cystine/unit of hexosaminidase activity, were prepared and cystine egress was determined as an initial velocity (pmol of half-cystine/min per unit of hexosaminidase), as previously described (Gahl *et al.*, 1982a). Corrections for rupture of lysosomes were made by measurement of soluble hexosaminidase activity, which averaged 7% of total hexosaminidase activity at zero time and 14% of total at 20–40 min. Cystine was measured by the cystine-binding-protein method (Oshima *et al.*, 1974); hexosaminidase activity (EC 3.2.1.30; 1 unit = 1 nmol/min) was assayed fluorimetrically as previously described (Gahl *et al.*, 1982b).

Cystine counter-transport

This was measured as [^3H]cystine uptake into granular fractions loaded with non-radioactive cystine and expressed as c.p.m. of [^3H]cystine taken up/h per unit of hexosaminidase (Gahl *et al.*, 1983). Granular-fraction cystine loading was over 3 nmol of half-cystine/unit of hexosaminidase, to ensure that [^3H]cystine uptake would proceed at the maximal rate (Gahl *et al.*, 1983). Soluble hexosaminidase activity averaged 7% of total at zero time and 18% at 60 min. Added compounds were used at concentrations that did not substantially alter soluble hexosaminidase activity.

Results and discussion

It has been proposed that baseline cystine transport in isolated granular fractions at pH 7.0 might be mediated through proton-pump ATPase

activity in conjunction with contaminating amounts of ATP (Jonas *et al.*, 1982). Therefore, we examined the effects of inhibitors of lysosomal acidification on cystine egress and counter-transport in the absence of added MgATP. NEM, a blocking agent for free thiol groups and a known inhibitor of proton-pump ATPase activity (Ohkuma *et al.*, 1982; Galloway *et al.*, 1983; Stone *et al.*, 1983), did not decrease the rate of cystine egress at pH 7.0. This was true whether cystine transport was calculated by the method of Jonas *et al.* (1982), as the change in granular-fraction cystine per unit of hexosaminidase after 20 min (results not shown), or as a velocity of cystine egress (Gahl *et al.*, 1982a), which was 23.7 pmol/min per hexosaminidase unit when no NEM was present and 24.0 when 5 mM-NEM was present. Two other inhibitors of ATP-dependent acidification, DCCD and the anion-transport inhibitor DIDS (Schneider, 1981; Stone *et al.*, 1983; Xie *et al.*, 1983), as well as the protonophore CCCP, failed to decrease cystine egress or counter-transport (Table 1). Neither did the weak bases NH_4Cl or methylamine hydrochloride, which dissipate proton gradients across lysosomal membranes (Table 1).

It appeared that the movement of cystine out of (egress) and into (counter-transport) normal lysosomes at pH 7.0 in the absence of added MgATP was not altered by inhibitors of acidification. We next investigated whether these inhibitors would diminish cystine transport in the presence of 2 mM exogenous MgATP. Initial experiments indicated that the stimulatory effect of MgATP required K^+ ; neither 2 mM- $\text{MgCl}_2/\text{MgATP}$ nor 90 mM-KCl alone stimulated cystine egress (Table 2), but together they produced a 2-fold increase in the velocity of cystine transport ($P < 0.01$, Student's *t* test). This $\text{MgCl}_2/\text{MgATP}/\text{KCl}$ effect was not

Table 1. Effects of DIDS, DCCD, CCCP, NH_4Cl and methylamine hydrochloride on granular-fraction cystine egress and exchange at pH 7.0

Normal leucocytes were loaded to over 5 nmol of half-cystine/unit of hexosaminidase and granular fractions were suspended in 0.25 M-sucrose/10 mM-Hepes/1 mM-NEM, pH 7.0. Results are means $\pm$ S.D. for *n* experiments each performed in duplicate on separate days.

Addition to medium	Egress velocity (pmol of half-cystine/min per unit of hexosaminidase)			$10^{-3} \times [\text{H}]$ Cystine uptake (c.p.m./h per unit of hexosaminidase)		
	Mean	S.D.	<i>n</i>	Mean	S.D.	<i>n</i>
None	31.5	6.2	9	7.8*	2.7	8
DIDS (100 μM)	32.7	10.0	2	9.9	2.4	2
DCCD (25 μM)	39.2	7.6	2	—	—	—
CCCP (25 μM)	25.0	12.9	3	7.6	1.7	4
NH_4Cl (10 mM)	27.1	0.1	2	6.1	1.0	2
$\text{CH}_3\text{NH}_3\text{Cl}$ (10 mM)	26.5	6.3	2	6.2	0.6	2

* 13.0 pmol of cystine/h per unit of hexosaminidase.

Table 2. *Effects of KCl, MgCl₂/MgATP, DIDS, DCCD, CCCP and NH₄Cl on cystine egress from normal lysosome-rich leucocyte granular fractions at pH 7.0*

Normal leucocyte granular fractions were loaded to over 4 nmol of half-cystine/unit of hexosaminidase by exposing the whole cells to 1 mM-cystine dimethyl ester. Granular fractions were suspended in 0.25 M-sucrose/10 mM-Hepes/1 mM-NEM, pH 7.0, containing the listed additions. KCl was 90 mM, MgCl₂ and MgATP 2 mM each, CCCP 20 μ M, NH₄Cl 40 mM, DIDS 100 μ M and DCCD 50 μ M. The MgATP solution was adjusted to pH 7.0 before addition. Egress velocity was calculated as described in the Experimental section. Results are means of *n* experiments each performed in duplicate on separate days.

Addition to medium	Velocity of egress (pmol of half-cystine/min per unit of hexosaminidase)		
	Mean	S.D.	<i>n</i>
None	30.5	8.5	5
KCl	28.0	7.7	5
MgCl ₂ /MgATP	32.2	5.5	3
KCl + MgCl ₂ /MgATP	59.6	19.7	5
KCl + MgCl ₂ /MgATP + DIDS	51.0	17.5	2
KCl + MgCl ₂ /MgATP + DCCD	86.9	38.6	2
KCl + MgCl ₂ /MgATP + CCCP	71.1	20.4	2
KCl + MgCl ₂ /MgATP + NH ₄ Cl	64.1	16.8	2

mitigated by DIDS, DCCD or CCCP, nor by 40 mM-NH₄Cl (Table 2).

Therefore it appeared that the enhancement of cystine egress at pH 7.0 was mediated by a MgATP/KCl effect, which was not dependent on lysosomal acidification. Such a phenomenon might be related to the known stimulation of cystine egress by permeant K⁺ ions (Bashan *et al.*, 1984), although the role of MgATP in making the lysosomal membrane permeable to K⁺ ions is unknown.

Since a K⁺-independent MgATP effect might occur at an extralysosomal pH other than pH 7.0, pH curves were constructed in the absence of added K⁺. The pH optima for cystine egress (6.2, Fig. 1) and exchange (6.4, Fig. 2) were shifted to the left by 2 mM-MgCl₂ and 2 mM-MgATP. Thus MgCl₂/MgATP enhanced cystine transport maximally at pH 5.5; this phenomenon was further examined by using [³H]cystine counter-transport to determine if it was mediated by stimulation of proton-pump activity. First, the MgCl₂/MgATP effect was not inhibited by 50 mM-NH₄Cl, 50 mM-methylamine hydrochloride or 50 μ M-CCCP (results not shown). Second, the stimulatory effect of MgCl₂/MgATP appeared to be due to Mg²⁺ rather than ATP. Whereas NaGTP, NaADP or Na₂ATP did not enhance cystine transport, 2 mM-MgCl₂ alone stimulated [³H]cystine uptake to the same extent as MgATP, and the two together had a less than additive effect (Table 3). MgCl₂ alone stimulated [³H]cystine uptake in a concentration-dependent fashion, and this effect reached a plateau at MgCl₂ concentrations above 4 mM (Fig. 3). The bivalent-cation chelator Na₂EDTA at 1.8 mM, which itself had no effect on cystine counter-transport, prevented the stimulatory effect

Fig. 1. *Velocity of cystine egress from normal leucocyte granular fractions at different pH values*

Cystine-loaded granular fractions, containing 5.3 and 16 nmol of half-cystine/unit of hexosaminidase, were placed in 0.25 M-sucrose/1 mM-NEM buffered with 25 mM-acetic acid/sodium acetate (pH 5.0–6.0), 25 mM-sodium phosphate (pH 6.0–7.0), or 10 mM-sodium Hepes (pH 6.0) (H). Samples were removed after 0 and 25–40 min at 37°C and cystine egress was determined. Points represent the means of three experiments performed on separate days in duplicate. ●, No addition; O, + 2 mM-MgCl₂/MgATP.

of 2 mM-MgCl₂ (Table 3). At 2 mM, Na₂ATP obliterated the stimulation of cystine counter-transport by 2 mM-MgCl₂, and increasing amounts of Na₂ATP (up to 9.2 mM) were unable to restore

Fig. 2. $[^3\text{H}]$ Cystine uptake by normal cystine-loaded granular fractions at different pH values
Normal granular fractions were loaded to 6.3nmol of half-cystine/unit of hexosaminidase and suspended in 0.25M-sucrose/1mM-NEM containing 6.6 μM - $[^3\text{H}]$ cystine. Buffers were 25mM-acetic acid/sodium acetate (A, pH 5.0–6.0) or 25mM-sodium phosphate (P, pH 6.0–7.0). $[^3\text{H}]$ Cystine uptake was expressed as c.p.m./h per unit of hexosaminidase. ●, No addition; ○, 2mM- $\text{MgCl}_2/\text{MgATP}$.

Fig. 3. Stimulation of MgCl_2 of $[^3\text{H}]$ cystine uptake into normal cystine-loaded granular fractions at pH 5.5
Normal leucocyte granular fractions loaded with cystine were suspended in 0.25M-sucrose/25mM-sodium acetate (pH 5.5)/1mM-NEM/7 μM - $[^3\text{H}]$ cystine, and different concentrations of MgCl_2 . $[^3\text{H}]$ Cystine uptake was expressed as percentage stimulation over control (no added MgCl_2). Results are from experiments performed on two separate days (▲ and ●).

Table 3. Effects of MgCl_2 and other compounds on normal granular-fraction cystine counter-transport at pH 5.5
Normal cystine-loaded leucocyte granular fractions were suspended in a medium of 0.25M-sucrose/25mM-sodium acetate, pH 5.5, containing 1mM-NEM, 7 μM - $[^3\text{H}]$ cystine and the listed additives adjusted to pH 5.5. Cystine counter-transport was measured as $[^3\text{H}]$ cystine uptake/h per unit of hexosaminidase. Experiments were performed in duplicate on different days, and results represent mean percentages of controls (no added compound) for each experiment.

Addition to medium	$[^3\text{H}]$ Cystine uptake (% of control)
None	100
MgATP (2mM)	168
NaGTP (2mM)	104
NaADP (2mM)	89
Na_2ATP (2mM)	102
MgCl_2 (2mM)	169
$\text{MgCl}_2 + \text{MgATP}$	192
Na_2EDTA (2mM)	108
$\text{Na}_2\text{EDTA} + \text{MgCl}_2$ (2mM)	91
MgCl_2 (2mM) + Na_2ATP (2mM)	83
$\text{MgCl}_2 + \text{Na}_2\text{ATP}$ (6mM)	99
$\text{MgCl}_2 + \text{Na}_2\text{ATP}$ (9mM)	95
MgCl_2 (4mM)	209
MnCl_2 (4mM)	222
MgSO_4 (4mM)	189

normal MgCl_2 stimulation. In addition, either 4mM- MgSO_4 or 4mM- MnCl_2 enhanced cystine counter-transport to approximately the same extent as did 4mM- MgCl_2 (Table 3). We conclude that the stimulation of cystine transport at pH 5.5 was a bivalent-cation effect not related specifically to ATP. One might speculate that the effect is due to a stabilization of the external lysosomal membrane, whose general function may be impaired at pH 5.5 compared with the more physiological pH 7.0.

We were unable to demonstrate a stimulatory effect of MgATP itself on cystine transport (egress or counter-transport) across polymorphonuclear-leucocyte lysosomal membranes at any pH. It appears reasonable that the special conditions the lysosomal cystine carrier sees, i.e. an acidic environment inside the lysosome and a neutral milieu in the cytoplasm, might make its activity prone to alteration by proton-pump ATPase activity (Jonas *et al.*, 1982, 1983). On the other hand, linking cystine transport to lysosomal acidification may waste the energy required to maintain an acid lysosome, since cystine will always move 'downhill' into the reducing environment of the cytoplasm. In any event, we conclude that the ATPase-dependent stimulation of cystine trans-

port seen in lymphoblast granules is not present in polymorphonuclear-leucocyte granules, whose cystine-transport system can be investigated without the addition of exogenous MgATP.

We acknowledge the long-standing contributions of Dr. Joseph D. Schulman to the study of cystinosis, and express appreciation for his continued interest.

References

- Bashan, N., Gahl, W. A., Tietze, F., Bernardini, I. & Schulman, J. D. (1984) *Biochem. Biophys. Acta* **777**, 267–273
- Gahl, W. A., Bashan, N., Tietze, F., Bernardini, I. & Schulman, J. D., (1982a) *Science* **217**, 1263–1265
- Gahl, W. A., Tietze, F., Bashan, N., Steinherz, R. & Schulman, J. D. (1982b) *J. Biol. Chem.* **257**, 9570–9575
- Gahl, W. A., Tietze, F., Bashan, N., Bernardini, I., Raiford, D. & Schulman, J. D. (1983) *Biochem. J.* **216**, 393–400
- Gahl, W. A., Bashan, N., Tietze, F. & Schulman, J. D. (1984) *Am. J. Hum. Genet.* **36**, 277–282
- Galloway, C. J., Dean, G. E., Marsh, M., Rudnick, G. & Mellman, I. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3334–3338
- Jonas, A. J., Smith, M. L. & Schneider, J. A. (1982) *J. Biol. Chem.* **257**, 13185–13188
- Jonas, A. J., Smith, M. L., Allison, W. S., Laiking, P. K., Greene, A. A. & Schneider, J. A. (1983) *J. Biol. Chem.* **258**, 11727–11730
- Ohkuma, S., Moriyama, Y. & Takano, T. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2758–2762
- Oshima, R. G., Willis, R. C., Furlong, C. E. & Schneider, J. A. (1974) *J. Biol. Chem.* **249**, 6033–6039
- Schneider, D. L. (1981) *J. Biol. Chem.* **256**, 3858–3864
- Schneider, J. A. & Schulman, J. D. (1982) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L. & Brown, M. S., eds.), 5th edn., pp. 1844–1866, McGraw-Hill, New York
- Stone, D. K., Xie, X. & Racker, E. (1983) *J. Biol. Chem.* **258**, 4059–4062
- Wilbrandt, W. & Rosenberg, T. (1961) *Pharmacol. Rev.* **13**, 109–183
- Xie, X., Stone, D. K. & Racker, E. (1983) *J. Biol. Chem.* **258**, 14834–14838