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THE EFFECT OF IONS AND IONOPHORES ON CYSTINE EGRESS FROM HUMAN LEUCOCYTE LYSOSOME-RICH GRANULAR FRACTION

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This paper describes the stimulation of exodus of cystine from lysosome-rich granular fractions by potassium. Potassium permeability into lysosomes is low, but in the presence of an ionophore or permeable anion, the movement of K^+ into lysosomes caused a large stimulation of cystine exodus. Lysosomal preparations from leucocytes of cystinotic patients, which lack carrier-mediated cystine transport, also manifested stimulation of cystine egress by valinomycin and K^+. This suggests that potassium-dependent cystine egress involves a carrier different from that defective in cystinosis, or occurs through a non-carrier-mediated mechanism.

Introduction

The enzymes of lysosomes can degrade susceptible proteins to amino acids and small peptides [1], which presumably then escape from the lysosomes into the cytoplasm. The ability of some amino acids, dipeptides and tripeptides to traverse the lysosomal membrane has been investigated by indirect methods [2–4]. Studies of lysosomal vacuolation in intact cells suggest that lysosomal membranes have some permeability to non-metabolizable amino acids with molecular weights less than 220000 [4]. In osmotic protection experiments, dipeptides (except when negatively charged) usually penetrate the lysosomal membrane more easily than free amino acids [3].

To study the movement of amino acids across lysosomal membranes, high concentrations of intralysosomal amino acids have been achieved by incubating cells or isolated lysosomes with amino acid methyl esters [2,5,6]. The methyl esters enter by diffusion across the lysosomal membrane, and then are hydrolyzed by intralysosomal hydrolases to the free amino acids. Reeves [5] had shown that amino acid accumulation in the presence of methyl ester appears to be a specific property of lysosomes. No accumulation could be detected in the nuclei mitochondria, sarcoplasmic reticulum or Golgi complex as a result of treatment with amino acid methyl ester [5,7].

Studies of lysosomes loaded by exposure to methyl esters suggest that the exit of some amino acids may be diffusional [5]. However, cystine exodus through membranes of human leucocyte lysosomal fraction appears to involve a carrier-

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid.

mediated mechanism which is impaired in cystinosis, a lethal human disorder marked by intralysosomal cystine accumulation [8]. The normal lysosomal cystine transport system is MgATP stimulated, and demonstrates saturation kinetics [8] and counter transport (trans-stimulation) [9]; these properties are not evident in cystinotic lysosomes.

The present report demonstrates that cystine exodus from isolated human leucocyte lysosomes is stimulated by potassium both in normal and cystinotic preparations, and is markedly influenced by certain ionophores.

Materials and Methods

Leucocyte loading. Leucocytes from approx. 150 ml of heparinized blood were prepared by Dextran sedimentation and hypotonic lysis of erythrocytes [6]. The leucocytes were suspended in Hanks' balanced salt solution (pH 7.0) and transferred to tubes containing the amino acid methyl ester, a methanolic stock solution of which had been taken to dryness by evaporating with nitrogen.

The lysosomes of intact leucocytes were loaded by exposure of the leucocytes to 1 mM cystine dimethyl ester as previously detailed [10]. Labelled methyl esters were prepared by methylation as described [6,10]. Cystine-loaded granular fraction were prepared as previously described [8]: after incubation with methyl ester the leucocytes were centrifuged at $300 \times g$ for 3 min, washed twice by suspension and recentrifugation in 0.25 M sucrose/10 mM Hepes (pH 7.0) at 4°C, suspended in 1.5 ml cold sucrose-Hepes and disrupted by sonication for 10 s using a Model W 140 cell disruptor with a microtip (Head Systems, Ultrasonic, Plain View, NY).

The supernatant of the 5 min $1000 \times g$ centrifugation was spun at $8500 \times g$ for 10 min and the pellet was washed once by suspension and recentrifugation in 2 ml fresh, cold sucrose buffer. This pellet represented the granular (lysosomal) fraction, and was consistently 3–4-fold enriched in cystine per mg protein compared to whole leucocytes. Lysosomal enzyme activity (hexosaminidase *a*) was enhanced 3–6-fold in the granular fraction.

The granular fraction was suspended in 95 mM KCl (or other salts as indicated) in 195 mM sucrose/10 mM Hepes (pH 7.0)/1 mM *N*-ethylmaleimide. Valinomycin (Sigma) or nigericin (Calbiochem-Behring), if added, were first solubilized in acetone; the same volume of acetone was added to controls. Incubation was at 37°C. At timed intervals, 1-ml aliquots were removed, centrifuged at $8500 \times g$ for 10 min at 4°C, and cystine was determined in the supernatant and in the lysosomal granular fraction pellet by the cystine-binding protein assay [11]. The exodus of cystine from the lysosome-rich granular fraction was matched by the appearance of cystine in the supernatant after the granular fraction had been separated by centrifugation, as had been shown before [5].

As a measure of lysosomal rupture during cystine efflux, hexosaminidase activity was assayed in the supernatant of each centrifuged aliquot, and compared with activity in the total aliquot in the presence of 0.1% Triton X-100. For assay, 100 μl protein suspension was incubated at 37°C for 3 min with 200 μl 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside (1.2 mM) in 13 mM citric acid/20 mM sodium phosphate (pH 4.4)/0.25 M sucrose. For measurement of nonlatent hexosaminidase, 100 μl lysosomes in medium were incubated without Triton for 2 min. One unit of hexosaminidase hydrolyzed 1 nmol substrate per min at 37°C.

The velocity of cystine exodus from lysosomes, corrected for lysosomal damage as measured by hexosaminidase release, was calculated. Since only the lysosomes in the granular fraction contains cystine, we demonstrate the efflux rate as pmol 1/2-cystine per unit hexosaminidase activity (which is a characteristic lysosomal enzyme), as has been described before [8–10].

Exodus of radioactive cystine, cysteine-*N*-ethylmaleimide, tryptophan and methionine from loaded lysosomes granule fractions was measured by high-voltage electrophoresis as described [10]. Protein was measured as described by Lowry et al. [12].

Due to variability among different lysosomal preparations, the results in the figures represent individual experiments; each experiment was performed at least three times with concordant outcome.

Results

In the presence of KCl the rate of cystine exodus from loaded granular fractions corrected for hexosaminidase release was increased about 5-fold by 1 μM valinomycin, an ionophore that selectively promotes K^+ permeability in biological membranes [13,14]. The effect of valinomycin on cystine egress required potassium ion in the medium. Little or no effect of valinomycin on cystine egress was noted when NaCl was substituted for KCl (Fig. 1).

The enhancement of cystine exodus by valinomycin in the presence of KCl cannot be ascribed to lysosomal disruption as measured by soluble hexosaminidase activity. When granular fractions were incubated for 10 min in 95 mM KCl 125 mM sucrose, the soluble hexosaminidase increased from 7 to 15%, in the presence of 1 μM valinomycin for 5 min, it increased from 7 to 14% (Fig. 2); this was also true when NaCl was substituted for KCl, or in the absence of added salts. Thus, the ionophore appeared to produce a negligible or small degree of lysosomal disruption, much too slight to account for the consistent, marked K^+-dependent activation of the rate of cystine escape (Fig. 2).

The rate of cystine exodus in the presence of 1 μM valinomycin was temperature-dependent, with a Q_{10} of 2.5 (data not shown).

By increasing the external osmolarity from 125 to 1000 mosM by the addition of sucrose, the rate of cystine release was slightly decreased, as would be predicted for osmotically active vesicles. However, the substantial enhancement of cystine release by potassium ion plus valinomycin remained (Fig. 3).

The disposal of certain other amino acids was also measured from normal leucocyte granular fractions obtained from whole cells loaded by exposure to 1 mM of the corresponding [^{3}H]methyl

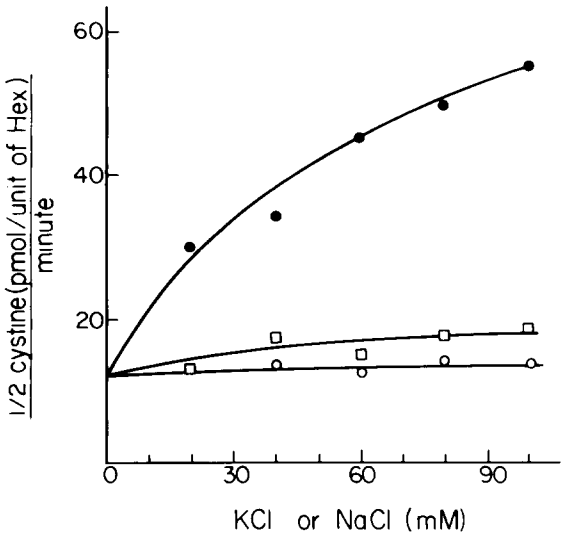

Fig. 1. The effect of valinomycin on cystine efflux from normal lysosomes-rich granular fraction in the presence of KCl and NaCl. Loaded lysosomal-rich granular fractions were prepared as described in Materials and Methods. 0.1 ml granular fraction suspension (0.1–0.3 mg protein) was added to 4 ml of different concentration of KCl in the absence (○———○) or presence (●———●) of 1 μM valinomycin or NaCl (□———□) in the presence of 1 μM valinomycin. The osmolarity was corrected to 315 mOsm by adding sucrose. Each tube contained 1 mM *N*-ethylmaleimide and 10 mM Hepes buffer (pH 7.4). The appearance of cystine in the supernatant was linear for 10 min with KCl + valinomycin and for 1 h with NaCl + valinomycin or with KCl. The rate of cystine efflux was measured also as described, Hex, hexosaminidase.

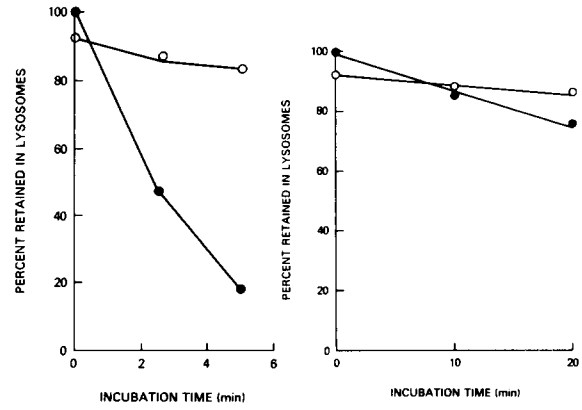

Fig. 2. Effect of valinomycin on cystine concentrations (●———●) and on hexosaminidase activity (○———○) remaining in lysosomes-rich granular fractions. Normal leucocytes were loaded with cystine using 1 mM cystine dimethyl ester and the granular fractions were obtained as described in Materials and Methods. The lysosomal-enriched fractions (0.1 ml) were incubated in sucrose-KCl in the presence (left) or absence (right) of 1 μM valinomycin (final volume 4 ml). At indicated time intervals, 100-μl aliquots were removed for measurement of total hexosaminidase activity. 1-ml aliquots were centrifuged and cystine content (percentage of zero-time value) and hexosaminidase activity (percentage of total) were measured in the lysosomal pellet.

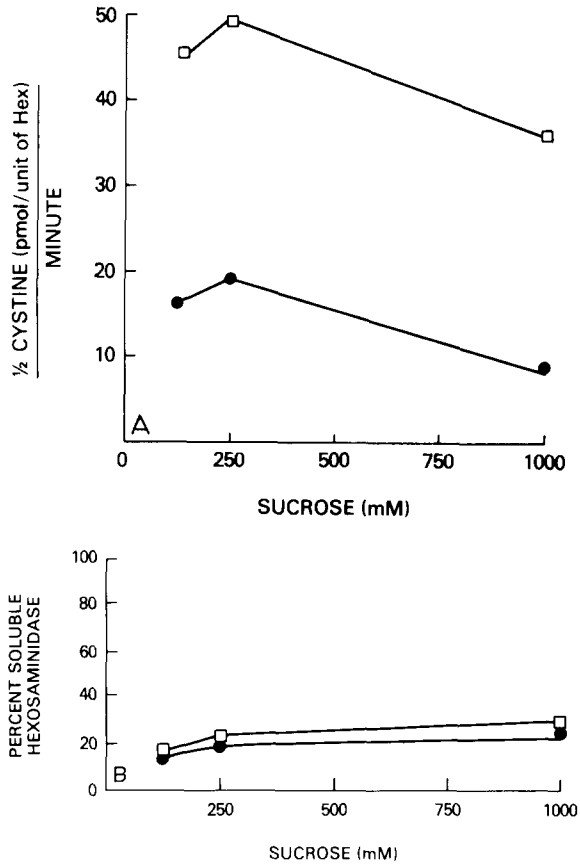

Fig. 3. Effect of medium osmolarity on cystine exodus from leucocyte granular fractions in the absence (●———●) and presence (□———□) of 1 μM valinomycin. Loaded granular fractions were prepared and incubated at 37 °C. Aliquots were centrifuged and their supernatant and pellets were assayed for cystine (A) and hexosaminidase (Hex) activity (B) as described in Materials and Methods. The medium contained 10 mM sodium-Hepes (pH 7.0), 90 mM KCl, 1 mM *N*-ethylmaleimide, and the sucrose concentrations indicated.

ester (Table I). Valinomycin together with potassium ion caused no significant increase in exodus of either tryptophan or methionine. In contrast, valinomycin and K^+ did cause substantial increases in the rates of exodus of cysteine-*N*-ethylmaleimide (Table I).

The release of cystine from loaded granular fractions was also increased in the presence of KCl by 2 μM nigericin, an ionophore that promotes electroneutral exchange of monovalent cations across biological membranes [14] (Table II). Moreover, eniantin B, another potassium ionophore [13] gave similar results. On the other hand, CICCP

TABLE I

AMINO ACID EGRESS FROM THE NORMAL LYSOSOME-RICH GRANULAR FRACTION

Conditions as noted in Materials and Methods. Cysteine-NEM was measured by high-voltage electrophoresis after loading with cystine dimethyl ester as described [10]. hex., Hexosaminidase; NEM, *N*-ethylmaleimide.

Amino acid	Ester concn. (mM) used for loading	Granular fraction loading (nmol/mg protein)	Egress rate (pmol/U hex. per min)	
			no valinomycin	plus valinomycin
Methionine	1.0	19.0	45	31
Tryptophan	1.0	5.20	18	17
Cystine	1.0	25	23	390
Cysteine-NEM	1.0	17.2	81	340

(carbonyl-cyanide *m*-chlorophenyl hydrazone), a protonophore, had no effect on cystine exodus, nor did it diminish the striking effect of valinomycin and KCl (Table II).

The effect of the ionophores could be demonstrated not only on the isolated granular fractions but on lysosomes within the whole cells as well. Normal leucocytes, loaded with cystine using cystine dimethyl ester, were incubated in Hanks' solution in the absence or presence of 1 μM valinomycin or 1 μM nigericin (Fig. 4). The load-

TABLE II

EFFECT OF IONOPHORES ON CYSTINE EXODUS

Leucocytes were loaded with 1 mM cystine dimethyl ester. Results are mean ± S.D. of (*n*) determinations. hex., hexosaminidase.

Addition	Concn. (μM)	Egress rate (pmol 1/2-Cys/U hex. per min)	
		315 mM sucrose	95 mM KCl + 125 mM sucrose
None	–	16.2 ± 4.0 (10)	18.2 ± 4.2 (15)
Valinomycin	1	12.5 ± 3.5 (5)	112 ± 10 (12)
CICCP	1	16	13.1 ± 3 (4)
CICCP + valinomycin		16	93 ± 9.6 (4)
Nigericin	2	15	98 ± 9.9 (8)
Eniantin B	0.5	10	47.6 ± 6.9 (4)

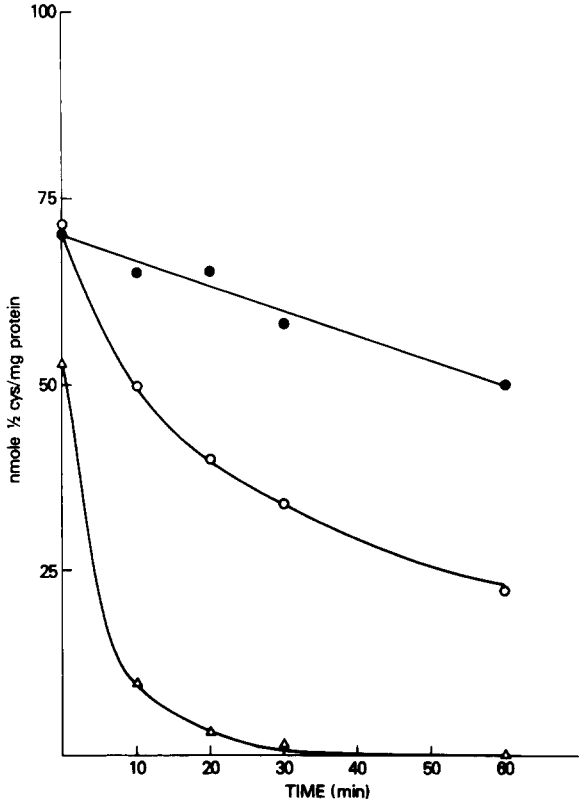

Fig. 4. Effect of valinomycin and nigericin on cystine exodus from normal human leucocytes. Leucocytes were loaded using 1 mM cystine dimethyl ester. After 30 min at 37 °C, the cells were washed three times with Hanks' solution by centrifugation at 900 × *g* for 10 min. 0.5 ml of cells were suspended in 6 ml Hanks' solution in the absence (●———●) or in the presence of 1 μM valinomycin (○———○) or 1 μM nigericin (△———△). At the time indicated, a 1-ml aliquot was removed. The leucocytes were washed again three times in Hanks' solution. Protein and cystine were measured as described [10,11]. Viability was measured with 0.3% Trypan blue, and was 94% after 1 h of incubation in all leucocyte preparations.

ing conditions increased intralysosomal cystine content in situ [6]. The rate of loss of cystine from the cells was increased by valinomycin and to an even greater extent by nigericin. The percent nonviability during the incubation, determined by exclusion of 0.3% Trypan blue dye, was not altered by the ionophores.

The effect of valinomycin plus potassium ion on promotion of cystine egress was observed not only in normal samples but also in cystine-loaded lysosome-rich granular fractions from four different cystinotic subjects (Table III).

TABLE III

CYSTINE EGRESS FROM LYSOSOME-RICH GRANULAR FRACTIONS OF CYSTINOTIC LEUCOCYTES

Cystinotic leucocytes were loaded with 0.25 mM cystine dimethyl ester and normals with 1 mM ester for 30 min to achieve more comparable levels of loading [10]. Granular fractions were incubated in 95 mM KCl/125 mM sucrose/1 mM *N*-ethylmaleimide/10 mM sodium-Hepes (pH 7.0). Samples were taken at 0 and 5 min for measurement of hexosaminidase (hex.) and cystine as described in Materials and Methods, and in Fig. 1.

	Egress rate (pmol 1/2-Cys/U hex. per min)		Cystine loading at zero time (pmol 1/2-Cys/U hex.)
	No addition	1 μM valinomycin	
Normals ($n = 9$)	18.2 ± 4.2	112 ± 10	1.8 ± 1.3
Cystinotics			
C.D.	0	222	1.2
D.S.	0	152	2.1
D.B.	0	114	3.7
J.C.	0	232	4.6

TABLE IV

EFFECT OF DIFFERENT CATIONS AND ANIONS ON CYSTINE EGRESS IN THE ABSENCE OF IONOPHORES

Leucocytes were loaded using 1 mM cystine dimethyl ester. The cystine-loaded lysosomes were incubated in 125 mM sucrose/95 mM salt/1 mM *N*-ethylmaleimide/10 mM sodium-Hepes (pH 7.0). Results are mean ± S.D. Where no S.D. is shown, the results are the mean of two concordant observations. hex., hexosaminidase.

Addition	Egress rate (pmol 1/2-Cys/U hex. per min)
Sucrose [a]	16.2 ± 4.0
KCl [a]	18.2 ± 4.2
KSCN [b]	50.2 ± 7.0
KI [b]	47.0 ± 6.8
K acetate [a]	16
K_2SO_4 [a]	10
NaI [a]	4
NaCl [a]	17.0

[a] Samples were taken at 0, 10 and 20 min to ensure linearity; calculations used 0 and 20 min points.

[b] Samples were taken at 0, 2.5 and 5 min to ensure linearity; calculations used 0 and 5 min points.

A common theme in the above results was promotion of egress of cystine from granular fraction under conditions which would be expected to enhance K^+ entry into the lysosomes. For this reason, we studied cystine egress from loaded granular fractions exposed to K^+ or Na^+ salts of various anions in the absence of ionophores. The order of anion permeability into membranes depends upon energy of hydration [15] and in rat-liver lysosomes is $SCN^- > I^- >$ acetate$^- > Cl^- \approx P_i \approx HCO_3^- > SO_4^{2-}$ [16].

Cystine exodus was markedly enhanced in the presence of the K^+ salts of penetrant anions (SCN^- and I^-), but not the less penetrant anions Cl^- and SO_4^{2-} (Table IV). Na^+ salts did not enhance cystine egress. The K^+ with penetrant anion effect was not due to lysosomal rupture, since soluble hexosaminidase increased with I^- and SCN^- by only 10–15%, whereas the increase in cystine exodus was 300%.

Discussion

The observations described above, appear compatible with the interpretation that potassium entry stimulates cystine exodus from human leucocyte lysosomes. Potassium entry could be effected by use of the ionophores valinomycin, nigericin or eniantin B (Table II) or by coupling potassium entry with that of a membrane-permeant anion (Table IV) in the presence of any ionophore.

Potassium-stimulated cystine egress in the presence of valinomycin was not a trivial phenomenon reflecting lysosomal damage. The effect of potassium entry upon hexosaminidase release from lysosomes was negligible in comparison to the marked enhancement of cystine egress from the lysosomes (Fig. 2). Furthermore, the magnitude of the valinomycin plus potassium effect was the same in osmotically protective high sucrose concentrations (up to 1 M) as in more isotonic buffer (Fig. 3). In addition, valinomycin and K^+ had no effect on the rates of exodus of methionine or tryptophan from the lysosome-rich granular fraction (Table I).

Potassium stimulation of cystine egress was specific, in that sodium could not substitute for K^+ (Fig. 1 and Table IV).

Potassium ion gradients support transmembrane potentials and may provide a driving force for gradient-coupled transport [17]. K^+ also interacts directly with membrane carriers in some systems, e.g., certain amino-acid carriers in *Escherichia coli* membrane vesicles [18], in insect midgut [19] and the Na^+-dependence L-glutamate carrier in renal plasma membranes [20]. In the latter system, Na^+-dependent L-glutamate uptake is stimulated by the presence of a $K_i > K_0$ gradient [21] and depends upon direct interaction of K^+ with the carrier [20]. In another system, internal K^+ stimulates the Na^+-dependent uptake of serotonin by platelet plasma membrane vesicles [22,23], and K^+ accelerates the conversion of the serotonin transporter from a form which releases internal substrates to one which binds external substrates.

The normal lysosomal cystine transport system appears to be carrier-mediated [8,9] but the potassium effect probably does not depend upon activity of this cystine transporter. Leucocyte lysosomes from cystinotic patients, who lack carrier-mediated cystine transport [8,10] and counter-transport [9], manifest a stimulation of cystine egress in the presence of valinomycin and potassium which is comparable to the stimulation in normal lysosomes (Table III).

One may speculate that a separate cystine carrier system could exist which depends upon potassium entry into lysosomes. * This system could be intact in cystinotics, but physiologically insignificant normal circumstances because of limited potassium entrance into lysosomes in vivo. Since valinomycin and potassium also stimulate cysteine-*N*-ethylmaleimide egress from lysosomal-rich granular fractions (Table I), a putative potassium-dependent cystine carrier system might not be specific for cystine, or other carrier systems might also be potassium-dependent.

Another possible mechanism for the potassium-dependent stimulation of cystine egress from lysosomes is via non-carrier-mediated transport; that is, potassium may interact with interior functional groups of the natural lysosomal membrane

* There is abundant evidence that certain amino acids are transported by several different carrier systems [24]; in particular, cystine uptake by fibroblasts is effected by multiple transport systems [25].

to selectively modify lysosomal permeability for certain amino acids.

Lysosomal cystine escape could, in theory, be electroneutral (involving movement of a counter ion) or electrogenic (resulting in a change in electrical potential which induces compensation at a separate membrane location. *

The potassium-dependent stimulation of lysosomal cystine egress appears to represent an electroneutral system. Valinomycin, which in the presence of $K_{out} > K_{in}$ gradient and chloride anion will change the internal lysosomal membrane potential in a net positive direction [13,14], increased the egress of cystine. However, nigericin, an electroneutral cation-exchange ionophore, also cause enhanced egress. Furthermore, the addition of the protonophore, CICCP, which should tend to dissipate the valinomycin-induced membrane potential, did not reduce or abolish the valinomycin plus potassium effect on cystine escape. Lastly, by using lipid-soluble anions like thiocyanate and iodide to replace the less penetrant chloride, and providing K^+ in the absence of ionophores, the electrical potential would be changed toward inside-negative [20], the opposite alteration to external KCl plus valinomycin. Under these conditions, there was still a potassium-dependent increase in the rate of cystine exodus. These diverse observations provide substantial evidence that the potassium effect, whatever its molecular mechanism, is not electrogenically mediated.

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* Most amino acid transport systems across biological membranes are electrogenic [26–28]. In contrast, there are transport systems, like that for Na^+ in the absence of glucose [11] or for glutamate in renal plasma membrane vesicles [20] which are voltage-insensitive, or electroneutral.