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ANION EFFECTS ON GLYCINE ENTRY INTO PIGEON RED BLOOD CELLS

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SUMMARY

(1) Glycine entry by the main Na^+-dependent route absolutely requires an anion as cofactor.

(2) Anion specificity is rather low. F^-, NO_3^-, HCO_2^-, SCN^- and I^- all replace Cl^- although they are less efficient than Cl^-. The K_m for glycine entry with NO_3^- or F^- replacing Cl^- is much higher than when Cl^- is the major anion. The K_m for glycine entry with SCN^- replacing Cl^- is similar to that with Cl^-.

(3) Acetate and larger anions do not satisfy the anion requirement.

(4) There appears to be an anion combining site on the glycine porter since, other than a limit on size, the activity order shows no correlation with simple physical or chemical properties of the anions.

(5) Only the K_m, not the V for glycine entry depends on Cl^- concentration. Thus Cl^- must combine with the porter before glycine can combine.

(6) When the internal Na^+ concentration is low, the internal Cl^- concentration does not effect V for glycine entry.

(7) Some anions allow a little glycine entry in the absence, but not the presence of the ASC amino acid transport inhibitor, alanine. This suggests that the ASC route of Christensen (see Eavenson, E. and Christensen, H. N. (1967) *J. Biol. Chem.* 242, 5386) either has a lower anion specificity than the main glycine route or lacks an anion requirement.

INTRODUCTION

Glycine movement across pigeon red cell membranes occurs by a Na^+-requiring route in which two Na^+ accompany each glycine and two Na^+ must combine with the glycine porter before glycine can (ref. 1 and citations therein, ref. 2). Small amounts of glycine cross by another Na^+-dependent route which carries chiefly small

Abbreviations: ASC route, the Na^+-dependent entry route characterized by Eavenson and Christensen[3], having alanine, serine and cysteine as typical good substrates. $[X]_{i,o}$ concentration of the substance indicated by the symbol inside ('i' subscript) or outside ('o' subscript); *e.g.* $[Cl^-]_o$: concentration of Cl^- in the medium; V, the limiting velocity of alanine-resistant, Na^+-dependent glycine entry as $[glycine]_o$ approaches infinity; K_m, the $[glycine]_o$ giving an alanine-resistant, Na^+-dependent glycine entry rate of $1/2\ V$. Subscripts 'i' or 'o' indicate the substance or process referred to is at the inside or outside of the cell, respectively. TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid.

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neutral amino acids (*e.g.* alanine, serine, cysteine), the ASC route of Christensen (see ref. 3). Some glycine movement also occurs by a Na^+-independent diffusion-like route[4].

It had been briefly reported[5] that glycine entry decreased as Cl^- in the incubation medium was progressively replaced by methanesulfonate and that this implied an anion requirement for glycine entry. The present paper concerns an examination of this anion requirement. We were chiefly concerned with anion effects on the main Na^+-dependent glycine entry route in these cells, that which is resistant to inhibition by alanine. The anion requirement is absolute but the specificity is fairly low. Several small anions not too similar to Cl^- can replace Cl^-. With some (F^-, NO_3^-) the K_m for glycine is greatly elevated. Anions larger than acetate do not replace Cl^-.

It is inferred indirectly that the ASC route either has a lower anion specificity or no anion requirement.

MATERIALS AND METHODS

Inorganic chemicals were reagent grade. [1-^{14}C]Glycine was obtained from New England Nuclear Corp., *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer from Calbiochem and D-glucose from Baker Chemical Co.

The organic anions were either purchased as sodium and potassium salts or prepared by neutralizing the free acids. They were generally the best grades from the listed sources. Sodium glycolate, hippuric acid and D,L-mandelic acid were from Matheson, Coleman and Bell; potassium acetate, benzoic acid and 85 % lactic acid (U.S.P.) were from Baker Chemical Co., 88 % formic acid and sodium acetate were from Malinkrodt Chemical Co.; potassium glycolate was from Aldrich Chemical Co.; *o*-bromobenzoic acid, cyclohexanecarboxylic acid, sodium isethionate, potassium isethionate, methanesulfonic acid and trifluoroacetic acid were from Eastman Kodak.

Sodium and potassium lactate stock solutions were prepared by diluting 85 % lactic acid to approx. 1 M, adding a slight excess of NaOH or KOH, boiling, titrating back to pH 7.2 with diluted (approx. 1 M) boiled lactic acid and diluting to volume.

Preparation of pigeon red cells, determination of Na^+-dependent glycine entry rates and Cl^- analyses were done as previously described[4,5]. Incubation media had 148 mM M^+A^-, where M^+ is either Na^+ or K^+ and A^- is the tested anion or an anion mixture totaling 148 mM, 6 mM TES, 3 mM sodium or potassium salts of TES (giving a total Na^+ or K^+ concentration of 151 mM), and 8.3 mM D-glucose. The concentrations of [^{14}C]glycine and unlabelled L-alanine are indicated in the figure legends and table footnotes.

To control the [Cl^-] in the ^{14}C-containing incubation media, it was necessary to first exchange cell Cl^- for the anion(s) to be tested. Exchange media were incubation media with K^+ as sole cation and no glycine or alanine. Where Cl^--free media were wanted, cells were given two successive 10-min incubations at 39 °C in 16.7 vol. of incubation medium with no Cl^- and 148 mM anion to be tested. Where some Cl^- was wanted in the final incubation medium, the internal [Cl^-] was adjusted by the appropriate exchange incubations whose time, cell/medium ratio and medium composition was calculated from exchange half times determined in preliminary experiments. For example, for a sample where lactate was to replace all but 15 mM Cl^- in the medium, the cells were incubated with 16.7 ml per g cells of Cl^--free medium for 15 min, 39 °C, a further 3.3 ml/g was added of medium containing 64 mM Cl^- and

84 mM lactate$^-$ and incubation continued 5 min more at 39 °C. After centrifugation, resuspension and incubation in the ^{14}C-containing medium (15 mM Cl^-, 132 mM lactate$^-$), cells and medium had 15 mM Cl^- as determined by Hg titration.

In general, except with SCN^- and I^-, the extent of exchange was verified by titration of medium and cell extracts with $Hg(NO_3)_2$. Adequacy of exchange of SCN^- and I^- was merely assumed.

Exchange of the larger anions, and also F^-, was slow in the cold so 39 °C exchange was required.

A small amount of Na^+-dependent glycine entry occurs by an alanine-inhibitable route, presumably the ASC route[3]. Alanine was added in all but the first survey experiments to suppress this component of Na^+-dependent glycine entry. "Glycine entry" will hereafter be used to mean the alanine-resistant Na^+-dependent glycine entry.

RESULTS

Existence of an anion requirement

Glycine entry has an absolute requirement for an anion. Upon complete replacement of Cl^- by any of several anions, glycine entry is negligible. This is not due to inhibition by these anions since rapid glycine entry occurs if a little Cl^- is furnished (lines 7–11, Table I).

Effect of various anions on Na^+-dependent glycine entry

A number of anions can replace Cl^- in supporting glycine entry. These are designated "active" anions in Table I. NO_3^-, SCN^- and F^- were selected for kinetic studies and will be discussed later.

Other anions allow some Na^+-dependent glycine entry in the absence of, but not in the presence of alanine, *e.g.* benzoate (Table I, line 13) and presumably others (acetate, lactate, mandelate, methanesulfonate and hippurate, lines 9–11, 14, 15, Table I). With the "active" anions, alanine appears to reduce Na^+-dependent glycine entry by about 15 % of the amount entering from 148 mM Cl^- medium (ref. 6 for Cl^- and Table I for the others). These data suggest that either the ASC route has a broad anion specificity or no anion requirement at all. We did not, however, directly test anion effects on entry of typical ASC substrates.

The larger anions cannot replace Cl^-. Some (Table I, lines 7–11) were tested in the presence of alanine with and without 15 mM Cl^-. These five anions which support no glycine entry but allow rapid glycine entry with 15 mM Cl^- are designated "inert". The not entirely justified implication (see Discussion) is that none of them combine with the glycine porter or otherwise effect it and therefore neither support transport nor inhibit its support by Cl^-. Some other incompletely tested anions may also fall in this class (lines 13–19, Table I).

Cyclohexanecarboxylate is clearly not inert, but its main effect is not to compete with Cl^-, since the $[Cl]_{\frac{1}{2}}$ value in its presence is similar to that with lactate, but rather to inhibit the maximum glycine entry rate with fixed $[glycine]_0$ and $[Na^+]_0$ and infinite $[Cl^-]_0$ (Fig. 1).

TABLE I

THE EFFECTIVENESS OF VARIOUS ANIONS IN REPLACING CHLORIDE

Internal Cl^- was exchanged for the anion in the medium as described in the text to eliminate contribution of Cl^- to the medium by exchange during the incubation with [^{14}C]glycine. External Cl^- was generally less than 0.5 mM as determined by titration of Cl^- except that no titration was done when SCN^- and I^- were used (text). Each entry number is the result from one experiment except where standard deviation of the mean (S.E.) values are given, where "*n*" indicates the number of experiments. Each number is the Na^+-dependent component of glycine entry in the presence of the tested anion(s), divided by the Na^+-dependent entry measured in the same experiment in the presence of Cl^- (148 mM), times 100. The Na^+ concentration was 151 mM and glycine generally 0.5 mM.

Anion replacing Cl⁻	*Glycine entry rate relative to that from Cl⁻ medium*			*Class**
	No alanine	*+ Alanine*	*+Alanine + 15 mM Cl⁻*	
1 Cl^-	100	(81% of "no alanine" entry rate)**	—	Active
2 F^-	50	19, 34, 33***	n.d.	
3 SCN^-	60	47, 46***	n.d.	
4 I^-	51, 51	n.d.	n.d.	
5 NO_3^-	65	23, 50, 52***	n.d.	
6 HCO_2^-	44, 54	n.d.	n.d.	
7 SO_4^{2-}	n.d.	2.2 ± 0.73 (± S.E., $n = 4$)	104, 99	Inert
8 Isethionate$^-$	n.d.	5.5 ± 1.5(± S.E., $n = 4$)	96, 99	
9 Acetate$^-$	13	5.7, 7.0	84, 86	
10 Lactate$^-$	14, 46(?)	2.5 ± 0.95(S.E., $n = 5$)	81 ± 3.3(S.E., $n = 4$)	
11 Mandelate$^-$	7	2.3, 1.7	68, 63	
12 Cyclohexane carboxylate$^-$	5	n.d.	31 ± 4.3(S.E., $n = 3$)	Inhibitory
13 Benzoate$^-$	16, 26	1.9, 3.6	n.d.	Inert in the presence
14 $CH_3SO_3^-$	18, 28	3.4	n.d.	but not absence of alanine
15 Hippurate$^-$	13	2.3, 1.7	n.d.	
16 Glycerate$^-$	n.d.	1.5, 1.5	n.d.	Low activity, incompletely
17 Glycolate$^-$	n.d.	3.7	n.d.	classified
18 *o*-Bromobenzoate$^-$	6.2	n.d.	n.d.	
19 Trifluoroacetate$^-$	26, 41	n.d.	n.d.	

* For fuller description of these classes, see text.

** This is an average value from earlier work[6] of the entry ratio in the presence and absence of alanine. It is comparable to the ratio of values in the "+alanine" and "no alanine" column for the other anions.

*** These values were obtained by interpolation from the kinetic experiments illustrated by Fig. 3a, 3b.

Kinetic dependence of glycine entry on $[Cl^-]_0$

To determine the kinetic parameters for glycine entry, a truly inert anion is required. Lactate was chosen for this role. An inert anion is needed because with the total anion concentration held constant, the form of the equation for glycine entry rate as a function of, *e.g.* $[Cl^-]_0$, is the same whether the other anion is inert or a pure competitive inhibitor for $[Cl^-]_0$. A plot of 1/(glycine entry rate) *vs* $1/[Cl^-]_0$ with lactate as inert anion is shown in Fig. 1. The dependence appears to be on the first power of $1/[Cl^-]_0$ with $[Cl^-]_{1/2}$ values, at 0.5 mM $[glycine]_0$ and 151 mM $[Na^+]_0$, of 8.0 and 8.5 mM (two experiments). Fig. 1 also shows an analogous plot with cyclohexanecarboxylate as the other anion. With it, $[Cl^-]_{1/2}$ values were 10.0 and 11.4 mM. The inhibition by cyclohexanecarboxylate shown in Table I is therefore not due to its competition with Cl^- but to the rise in the intercept value, relative to lactate as major anion, of 2.5- and 2.7-fold in two experiments.

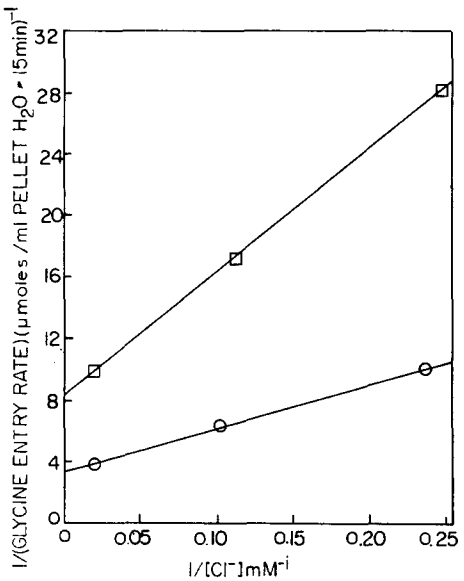

Fig. 1. Double reciprocal plots of alanine-resistant Na^+-dependent glycine entry rates against Cl^- concentration. Cell Cl^- was exchanged (Methods) for external anions to achieve internal $[Cl^-]$ values approximately equal to the external $[Cl^-]$ values shown on the abscissa. The cell samples were then incubated 15 min, 39 °C, with 0.5 mM [^{14}C]glycine and 5 mM unlabelled L-alanine in media with the indicated $[Cl^-]$ and sufficient lactate (lower line) or cyclohexanecarboxylate (upper line) to give [anion] + $[Cl^-]$ = 148 mM. Na^+-dependent uptake was obtained from total uptake from media with 151 mM Na^+ by subtracting uptake calculated from the glycine entry from Na^+-free (K^+ replaced Na^+) media with 0.5 mM [^{14}C]glycine, 5 mM L-alanine, 10 mM Cl^- and 138 mM lactate or 138 mM cyclohexanecarboxylate. Uptake is in μmoles/ml pellet water per 15 min at 39 °C. For this experiment, the intercept and $[Cl^-]_{1/2}$ values were 0.30 μmoles/ml per 15 min and 8.0 mM, and 0.12 μmoles/ml per 15 min and 10.1 mM for lactate and cyclohexanecarboxylate media, respectively.

Fig. 2a shows 1/(glycine entry rate) plotted against $1/[glycine]_0$ at several $[Cl^-]_0$ values. The K_m for glycine depends on $[Cl^-]_0$ while V does not. This means that Cl^- must combine with the transport mechanism before glycine can combine,

otherwise no increase in $[glycine]_0$ could overcome the limitation of low $[Cl^-]_0$ and V would depend on $[Cl^-]_0$ (see Discussion).

This same data was plotted as 1/(glycine entry rate) *vs* $1/[Cl^-]_0$ at several (interpolated) values of $[glycine]_0$ and should have given linear plots as in Fig. 1. For reasons we are totally at loss to find, these plots were slightly curved. In the two experiments of this sort (one is shown in Fig. 2b) linear relationships were found between 1/(glycine entry rate) and $1/[Cl^-]_0^{1.28}$ and $1/[Cl^-]_0^{1.24}$. Although only three $[Cl^-]$ points were used at any one glycine concentration, $[Cl^-]_{1/2}$ depends strongly on [glycine], so that linearity is found with four different sets of $[Cl^-]_{1/2}/[Cl^-]$ values. We conclude that the glycine entry rate depends on $[Cl^-]_0$ to approximately the first power, but whether it is exactly first power or not is unclear.

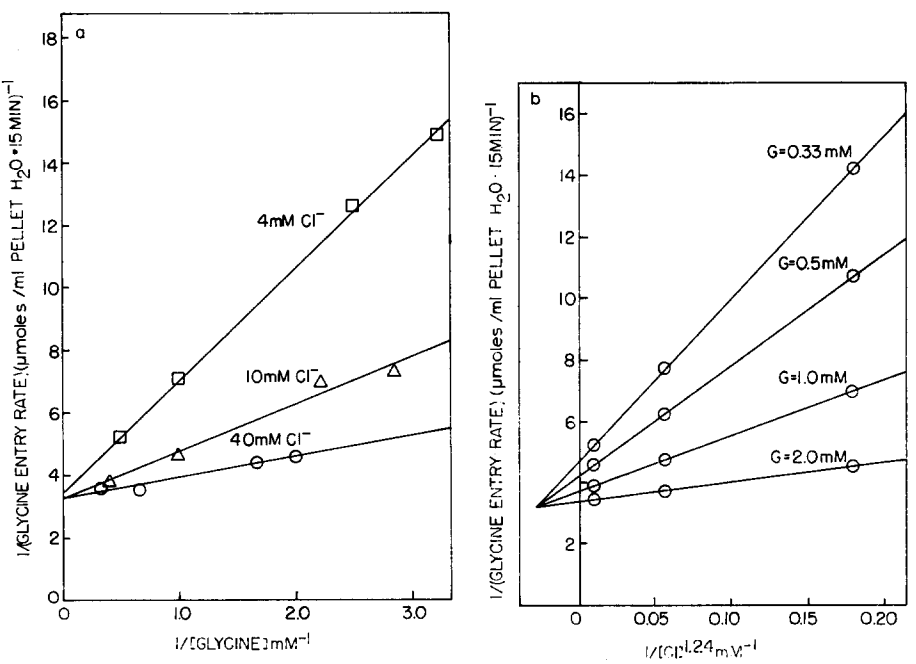

Fig. 2 (a) Double reciprocal plots of alanine-resistant Na^+-dependent glycine entry rates against glycine concentrations at three Cl^- concentrations. The experiments were done as for Fig. 1, lactate line, except that the glycine concentration was also varied as shown. A referee pointed out a slight bias in the drawing of one line in this figure. The intercept values obtained from the original plot, redrawn without bias, are 3.50, 3.06 and 3.26 at 4, 10 and 40 mM $[Cl^-]$, respectively, and the corresponding numbers for the duplicate of this experiment (not shown) are 3.00, 2.83 and 2.83. (b) Double reciprocal plots of alanine-resistant Na^+-dependent glycine entry rate against $[Cl^-]$ obtained from the data shown in (a). Glycine entry rates for 0.33, 0.5, 1.0 and 2.0 mM glycine were obtained by interpolation from the lines shown in (a) at the $[Cl^-]$ values used in the experiment. Plots of 1/(glycine entry rate) *vs* $1/[Cl^-]^x$ were made and the value of x giving a straight line was obtained by trial and error. The figure shows these plots with the value of x so obtained.

Because $[Cl^-]_i$ was adjusted by prior exchange to always equal $[Cl^-]_0$ we conclude (see Discussion) that V for glycine is not affected by $[Cl^-]_i$ when, as here, $[Na^+]_i$ is low.

Effects of replacement of Cl^- by active anions

The K_m and V values for glycine were determined with NO_3^-, SCN^- and F^- wholly replacing Cl^-. For direct comparison, in each of these experiments the V and K_m values were also determined with Cl^- as sole anion. These data are illustrated in Fig. 3a and b and the V and K_m values tabulated in Table II along with the ratios of

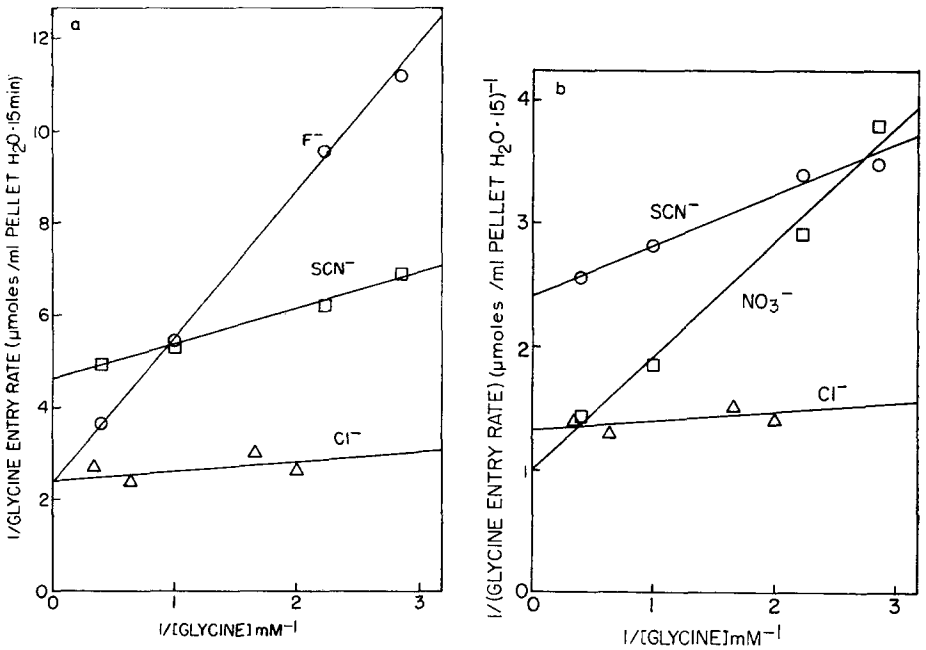

Fig. 3. Double reciprocal plots of alanine-resistant Na^+-dependent glycine entry rates against glycine concentration with F^-, SCN^- and NO_3^- replacing Cl^-. Experiments were done as for Fig. 2 except that where Cl^- was replaced, essentially all Cl^- was replaced by F^- or SCN^-. For the experiment shown in (a), V values, in μmoles/ml pellet water in 15 min, 39 °C, were 0.41, 0.22 and 0.43 for Cl^-, SCN and F^- media, respectively, while K_m values in the same order were 0.08, 0.17 and 1.36 mM. For the experiment shown in (b), V values were 0.76, 1.0 and 0.42 for Cl^-, NO_3^- and SCN^- media and K_m values in the same order were 0.06, 0.93 and 0.17.

TABLE II

THE V AND K_m VALUES FOR ALANINE-INDEPENDENT Na^+-DEPENDENT GLYCINE ENTRY WITH VARIOUS ANIONS REPLACING Cl^-

The V and K_m values were estimated from double reciprocal plots illustrated by Figs 3a and 3b. Values without subscripts are for glycine entry from media where Cl^- was replaced by the listed anion. The ratios, V_A/V_{Cl} and K_{mA}/K_{mCl} are the ratios of the values from the test anion media to the values from Cl^- media measured in the same experiment.

Anion	*Number of values averaged*	$V \pm$ S.E. (*μmoles/ml pellet water·15 min*)	$K_m \pm$ S.E. (*mM*)	$\frac{V_A}{V_{Cl}} \pm$S.E.	$\frac{K_{mA}}{K_{mCl}} \pm$ S.E.
Cl^-	4	0.433 ± 0.115	0.078 ± 0.013	≡ 1	≡ 1
F^-	3	0.302 ± 0.076	1.377 ± 0.112	0.905 ± 0.154	17.10 ± 2.03
SCN^-	2	0.317 ± 0.100	0.170 ± 0.002	0.537 ± 0.011	2.47 ± 0.46
NO_3^-	3	0.616 ± 0.189	2.144 ± 1.174	1.507 ± 0.192	24.33 ± 7.86

V and K_m values to those with Cl^- as sole anion as determined in the same experiment. (However, see Discussion, the K_m values with Cl^- are not accurate.) With F^- and NO_3^-, K_m values are much higher than with Cl^-, while with SCN^- the K_m is similar. With SCN^- the V for glycine entry is approx. 1/2 that with Cl^-, with F^- it is similar, while with NO_3^- it is higher.

Na^+-independent glycine entry

Anion effects on Na^+-independent glycine entry were erratic, but in all experiments such entry from Cl^- medium was greater than that from any other anion medium used in the same experiment. For example, the ratio: entry from lactate media/entry from Cl^- media was 0.46 ± 0.33 (S.E.); ± 0.15 (S.E.), $n = 5$. This, like the earlier observation of β-phenylethylamine inhibition of Na^+-independent glycine entry[7] suggests that Na^+-independent glycine entry is a mediated process and not simple diffusion.

DISCUSSION

There is an absolute requirement for an anion for glycine transport by the main (alanine-resistant, Na^+-dependent) glycine transport route since several anions fail to replace Cl^- but do not inhibit glycine entry when a small amount of Cl^- is furnished.

Anions might combine with the glycine porter in place of Cl^- and function like it ("active" anions), combine but not function ("competitive inhibitors"), or not combine and therefore not function, and also not act by any other mechanism ("inert" anions). We found five "active" anions. (Br^-, which was not studied here, had been previously found to be active (G. A. Vidaver, unpublished).) "Active" anions do not obviously share any property but small size. Table III lists crystal radii and

TABLE III

ANION RADII

Stokes radii, effective hydrated radii and crystal radii indicated by * were taken from Nightingale[9]. Stokes and effective hydrated radii indicated by † were calculated by us as described by Nightingale from limiting ionic conductance values from the International Critical Tables.

Anion	*Uncorrected Stokes radius (Å)*	*Effective hydrated anion radius (Å)*	*Crystal radius*
Br^-	1.18*	3.30*	1.95*
I^-	1.19*	3.31*	2.16*
Cl^-	1.21*	3.32*	1.81*
NO_3^-	1.39*	3.35*	2.64*
SCN^-	1.40†	3.39†	
F^-	1.66*	3.52*	1.36*
Formate$^-$	1.69†	3.52†	
Acetate$^-$	2.25†	3.76†	
SO_4^{2-}	2.30*	3.79*	2.90*
Lactate$^-$	2.63†	3.94†	
o-Bromobenzoate$^-$	3.80†	4.01†	
Benzoate$^-$	2.83†	4.02†	
Mandelate$^-$	3.18†	4.18†	
Hippurate$^-$	3.54†	4.34†	

"effective" hydrated anion radii. Anions with "effective" hydrated radii > about 3.7 Å do not replace Cl^-. Typical halogen anions are active (Cl^-, SCN^-, I^-) but so is F^- which is chemically unlike the other halogens. Also active are NO_3^- and HCO_2^-. The activity order does not correspond to the chaotropic series, similar to the Hofmeister series, which is $SO_4^{2-} < CH_3COO^- < Cl^- < Br^- < NO_3^- < ClO_4^- < I^- < CNS^-$ (protein destabilization)[8]; $SO_4^{2-} < CO_3^{2-} < F^- < Cl^- < Br^- < ClO_4^- < SCN^-$ (polyvinyloxazolidone cloud point)[8]. Thus Cl^- and SCN^-, near opposite ends of the series, give the most similar K_m (glycine) values and F^-, to the left of Cl^-, behaves like NO_3^- which is to the right of Cl^-. Among the active anions other than Cl^-, there is no obvious correlation between activity and position in the series.

Since two Na^+ are co-transported with each glycine[1], it might have been supposed that the anion merely forms an ion pair thus reducing charge separation during the translocation step which may carry Na^+ through a low dielectric strength environment. While this may still be the function of the anion, the lack of correlation between activity and any simple physical property implies the existence of an actual anion combining site.

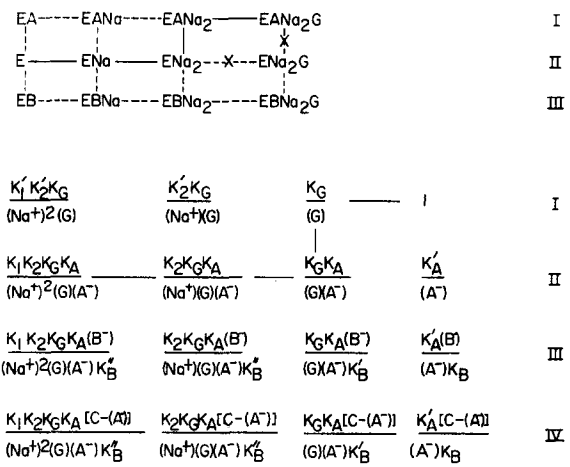

Fig. 4. The various forms of externally oriented glycine porter with two anions present and the terms in the equation for glycine entry rate arising from them. It is assumed that the various forms are in equilibrium with each other. [G], [Na^+], [A^-] and [B^-] refer to concentrations of glycine, Na^+, and the anions A^- and B^-, respectively. The total anion concentration, C, is constant. K is used to indicate dissociation constants with the subscripts designating the species dissociating. Primes and double primes differentiate non-equivalent dissociation constants. The number of independent dissociation constants is smaller than the number shown. For our purposes it is not necessary to eliminate redundant constants. The solid lines merely connect the forms and terms related by the unprimed constants. On the basis of the data reported in this paper, the lines with × across them represent reactions which do not occur.

No pure competitive inhibitor was found (although we did not look very hard). While cyclohexanecarboxylate is inhibitory, it is at best weakly competitive with Cl^- (Fig. 1). It may belong to the same rather nondescript class of inhibitors as β-phenylethylamine, octanoate, *etc.*[7]. β-Phenylethylamine is a non-competitive inhibitor[7].

Lactate, acetate, mandelate, isethionate and SO_4^{2-} are designated "inert". That a given anion is inert can only be established if it is one of a group of inert anions. If an active anion and a competitive inhibitor anion are both present and the sum of

their concentrations is kept constant, the equation for glycine entry rate as a function of the active anion concentration will have the same form regardless of the dissociation constant of the competing anion. However, the numerical values of the parameters describing glycine entry rate dependence on active anion concentration will depend on the affinity of the inhibiting anion. The independence of the form of the glycine entry rate equation on whether the second anion is inert or a competitive inhibitor for the active anion can be seen from Fig. 4. The upper lines I–III give the complexes of externally oriented glycine porter where A is an active anion and B an anion which can combine but whose complex, $E\mathrm{BNa_2G}$, cannot reorient. "B" is thus a competitive inhibitor. The lower lines I–III give the corresponding expressions for the complexes in terms of dissociation constants and concentrations. When the total anion concentration, C, is constant, the lower line III becomes line IV, where each term in line III is split into a pair, one member of which, the term with "C", can be added to the corresponding term in line II and the other member, where (A) in the numerator and denominator cancel, can be added to the corresponding term in line I. This sum of terms has the same form whether K_B is finite or not. This sum of terms, with each term multiplied by the appropriate constant is the equation for the reciprocal of the glycine entry rate. Thus the form of the glycine entry rate equation cannot reveal inhibition by anion "B". Chemically different competing anions ("B") should, however, give different dependencies of glycine entry rate on $[A^-]$. If there is a group of chemically dissimilar anions giving the same numerical value for this dependence, their affinities are presumably all very low, *i.e.* they are all inert.

The experiments of Figs 1 and 2 were done with lactate as "inert" anion. This choice was made before the observations that SO_4^{2-} or isethionate *plus* 15 mM Cl^- gave as rapid glycine entry as 148 mM Cl^- alone. It had been assumed that lactate, acetate and mandelate were sufficiently different that the near equivalence of the glycine entry rates with any of these *plus* 15 mM Cl^-, together with the fact that these were less than the rates with 148 mM Cl^-, meant that none of these anions competed significantly with Cl^-. The SO_4^{2-} and isethionate data do not fit this interpretation. However, preliminary experiments indicate that during the 39 °C preincubation to exchange internal Cl^- for the test anion mixture, some glycine transport capacity is lost (20–30 %) when preincubation is with lactate, acetate, mandelate, Cl^- or SCN^-, but less or none when it is with SO_4^{2-}, isethionate or NO_3^-. That is, the difference between entry rates with isethionate or SO_4^{2-} *plus* 15 mM Cl^- and with acetate, lactate or mandelate *plus* 15 mM Cl^- has an explanation other than inhibition by the latter anions. There is another reason for supposing lactate does not combine with the glycine porter and is therefore inert. The very different anions lactate and cyclohexanecarboxylate give quite similar $[Cl^-]_{\frac{1}{2}}$ values implying low affinity of the glycine porter for both.

Even if lactate is not inert, the only conclusion affected would be the relationship between the numerical value for $[Cl^-]_{\frac{1}{2}}$ and the affinity of the glycine porter for Cl^-. The other conclusions, *i.e.* the absolute requirement for an anion, the requirement for combination of an anion with the glycine porter before glycine can combine, the approximate first power dependence on $[Cl^-]$ and the independence of V (glycine entry) on internal or external $[Cl^-]$, all arise from the form of the dependence of glycine entry rate on $[Cl^-]_0$, not the numerical value for $[Cl^-]_{\frac{1}{2}}$.

From the kinetic behavior we conclude that Cl_0^- must combine with the glycine

porter before glycine can combine, and that V is independent of $[Cl^-]_i$. In the experiments from which these conclusions are drawn $[Cl^-]_i = [Cl^-]_o$ due to the prior exchange step, so effects of internal and external $[Cl^-]$ must be disentangled.

Since V, the limiting entry rate as [glycine] approaches infinity, is $[Cl^-]$ independent (Fig. 2a), the dependence of entry rate on $[Cl^-]$ arises from a $[Cl^-]$ dependence of K_m. As there appears to be no $[Cl^-]$independent component of entry rate (Figs 1 and 2a) and hence of K_m, $E\mathrm{ClNa_2G_o}$ ($E\mathrm{ANa_2G}$ of Fig. 4) is the only productive complex. Referring to Fig. 4, top two lines, there are two possibilities: (1) $E\mathrm{Na_2G_o}$ is absent, *i.e.* there is an obligate binding order of Cl^- first, then glycine, or (2) $k_3/k_3' \ll 1$, where k_3 and k_3' are defined by

$$E\mathrm{Na_2G_o} \xrightarrow{k_3} E\mathrm{Na_2G_i} \quad \text{and} \quad E\mathrm{ClNa_2G_o} \xrightarrow{k_3'} E\mathrm{ClNa_2G_i}.$$

In the second case, however, again referring to Fig. 4, with $[\text{glycine}]_o$ approaching infinity and finite $[Cl^-]_o$, $E\mathrm{Na_2G_o}$ and $E\mathrm{Na_2G_o}$ would coexist and increasing $[Cl^-]_o$ would shift the balance toward the productive complex $E\mathrm{ClNa_2G_o}$. That is, were there no obligate order of combination, first Cl^-, then glycine, V should be a function of $[Cl^-]$ just as the limiting velocity with $[Cl^-]$ approaching infinity and $[\text{glycine}]_o$ finite is a function of $[\text{glycine}]_o$ (Fig. 2b). With this obligate binding order and $[\text{glycine}]_o$ approaching infinity all externally oriented porter is in the form $E\mathrm{ClNa_2G_o}$ and the external-to-internal reorientation rate is $(E\mathrm{ClNa_2G_o})k_3'$ and is independent of $[Cl^-]$.

The other step at which $[Cl^-]$ might affect V is the inside-to-outside reorientation step or steps:

$$E_i \xrightarrow{k_{-o}} E_o \quad \text{and} \quad E\mathrm{Cl}_i \xrightarrow{k_{-o}'} E\mathrm{Cl}_o.$$

From previous kinetic studies[1] complexes other than E_i and $E\mathrm{Cl}_i$ should be negligible when $[Na^+]_i$ is low, and at high $[Cl^-]$, k_{-o} and/or k_{-o}' are at least as small and probably considerably smaller than k_3'. (The constant k_3' here corresponds to k_3 in ref. 1.) But then for V to be $[Cl^-]$ independent, the inside-to-outside reorientation must also be $[Cl]_i$ independent over the $[Cl^-]$ range used here. That is, V is independent of both $[Cl^-]_i$ and $[Cl^-]_o$ because both steps contributing to V are independent of the $[Cl^-]$ on their respective sides of the membrane.

In the discussion above, we tacitly assumed Cl^- to be co-transported with glycine but this is not essential to the argument. There is no direct evidence for co-transport of Cl^- and glycine. Since the membrane is permeable to Cl^-, coupling between glycine and Cl^- fluxes cannot be directly measured.

In earlier work (Table I of ref. 10) the flux stoichiometry between Na^+ and glycine was similar at high and low $[Cl^-]$.

The anion requirement for the ASC route was not directly determined. If the small alanine-inhibitable component of Na^+-dependent glycine entry is due to the ASC route, the activity of some anions, *e.g.* benzoate, in allowing alanine-inhibitable, but not alanine-uninhibitable glycine entry implies different anion specifities for the two routes. It is tempting to associate the requirement for the second Na^+ of the former with the anion requirement and to suppose the ASC route, kinetically dependent on the first power of $[Na^+]_o$, requires no anion.

Both K_m and V for glycine entry appear to depend on the nature of the anion (Figs 3a, 3b; Table II). For example, K_m values for glycine with F^- or NO_3^- as sole anion are much higher than when Cl^- is the sole anion. Although the relative K_m values are given in Table II, these cannot be very accurate* since the K_m values with Cl^- were determined with a range of glycine concentrations covering too small a portion of the saturation curve for accurate K_m determination. The glycine concentration ranges for F^- and NO_3^- were adequate however, so these K_m values must be much higher than that with Cl^-. With SCN^-, K_m appears somewhat higher than with Cl^- so its estimate is a little more reliable but still not accurate. We conclude only that K_m with SCN^- is similar to and possibly somewhat higher than that with Cl^-.

We conclude the K_m with NO_3^- is much higher than with Cl^- despite its very large standard deviation of the mean (S.E.). This large S.E. was due to one very high value for K_m with NO_3^- as anion. The three experiments with NO_3^- gave K_m values of 0.93, 0.96 and 4.5 mM. We think the very high value is aberrant since it was over 4.5 times as large as either of other two K_m (NO_3^-) values. This is a disagreement between replicate determinations far greater than we normally encounter, *e.g.* among the K_m values with SCN^- and F^- and the two lower values for NO_3^-, the largest ratio of replicate values is 1.33 and even among the relatively inaccurate K_m (Cl) values the largest ratio is 1.96. Also the K_m values near 1, but not 4.5 mM are compatible with the entry rate value, "no alanine", in Table I.

The V differences, although real, may not indicate differences in rate constants for the complexes containing Cl^-, SCN^- and NO_3^- ($E\text{Na}_2\text{G anion}_0 \rightarrow E\text{Na}_2\text{G anion}_1$) but rather reflect different losses in transport capacity during the preincubation anion exchange. This possibility has been discussed earlier in connection with "inert" anions.

One obvious exploitation of an anion binding site as a tool is the use of a chemically reactive "active" anion as an active site label. We attempted to use CNO^- this way. Although it was an effective irreversible inhibitor, this inhibition was not affected by replacing lactate by Cl^-.

In earlier work[5], replacement of Cl_0^- by the non-penetrating anion, mucate, reduced the glycine entry rate ("mucate inhibition") by both increasing K_m and decreasing V for glycine entry. Replacement of Cl_0^- by methanesulfonate also decreased the glycine entry rate, but apparently by less than replacement by mucate. The interpretation then made was that "mucate inhibition" was partially due to a specific Cl^- requirement and partially a consequence of the Donnan effect produced by the non-penetrating anion, while the methanesulfonate effect was due to a Cl^- requirement. On the basis of the present data, this interpretation would be extended by attributing the decrease in V seen in "mucate inhibition" to the Donnan effect and attributing the decrease in entry rate on partially replacing Cl^- by methanesulfonate to the anion requirement which methanesulfonate does not satisfy.

The relatively slight effect of replacement of Cl^- by acetate (an inert anion) or NO_3^- seen in Table I of ref. 5 is accounted for by Cl^- in those media (calculated

*When two or more K_m and V values are to be determined in one experiment and the values are very different, it is difficult to choose conditions allowing estimation of all with similar accuracy and still stay within the limitations required for reasonable initial rate approximations. In these experiments we chose to sacrifice accuracy of the K_m determination with Cl^-, since, as was found, we expected large K_m effects and wanted accurate V ratios.

concentration: approx. 13 mM) coming from cells. Those cells had not been pre-equilibrated with the incubation medium.

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REFERENCES

1 Vidaver, G. A. and Shepherd, S. L. (1968) *J. Biol. Chem.* 243, 6140.
2 Vidaver, G. A. (1971) *Biochim. Biophys. Acta* 233, 231.
3 Eavenson, E. and Christensen, H. N. (1967) *J. Biol. Chem.* 242, 5386.
4 Vidaver, G. A. (1964) *Biochemistry* 3, 662.
5 Vidaver, G. A. (1964) *Biochemistry* 3, 799.
6 Vidaver, G. A. Romain, L. F. and Haurowitz, F. (1964) *Arch. Biochem. Biophys.* 107, 82.
7 Kittams, D. W. and Vidaver, G. A. (1969) *Biochim. Biophys. Acta* 173, 540.
8 von Heppel, P. H. and Schleich, T (1969) *Acc. Chem. Res.* 2, 257.
9 Nightingale, E. R., Jr (1959) *J. Phys. Chem.* 63, 1381.
10 Vidaver, G. A. (1964) *Biochemistry* 3, 803.