in the bulk properties of the membrane (e.g., resistance to motion of molecules in it) due to polarization of dipolar molecules in it.

Fourth, replacement of Cl_o^- by mucate could also reduce glycine entry if there were a specific Cl^- requirement for formation of the glycine-containing complex (a K_m effect) or for its passage across the membrane (a V_{max} effect). Of the various possible mechanisms, the present work provides direct evidence only for the last.

Low concentrations of Cl_o^- seem more effective in relieving methanesulfonate inhibition than in relieving mucate inhibition (e.g., compare glycine entry at 10 mM Cl_o^- in Fig. 5 with that in Fig. 1 or 3). Although the point would have to be established by direct comparison at identical Na_o^+ and $glycine_o$ values, the difference is probably too great to be due solely to the differences in Na_o^+ and in the $glycine_o$/saline K_m ratios; therefore mucate inhibition includes, but is probably more than, a specific requirement for Cl_o^-.

The prediction from the hypothesis (see introductory paragraphs) was that the Donnan effect should reduce glycine entry relative to exit. This prediction applies to the special case (not attainable with intact cells) where Na_i^+ equals Na_o^+ and $glycine_i$ equals $glycine_o$. With the present experiments, only the effect on entry was measured. While the observed inhibition of entry is consistent with the hypothesis that the Na^+ gradient is the energy source for the glycine pump, it does not prove it.

ACKNOWLEDGMENTS

The author wishes to thank Professor Felix Haurowitz for his advice and support throughout the course of this work. He also wishes to thank Miss Pam Weedman, Miss Ann Kocher, and Mr. Roger Stickney for technical assistance.

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Some Tests of the Hypothesis that the Sodium-Ion Gradient Furnishes the Energy for Glycine-active Transport by Pigeon Red Cells*

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Received January 9, 1964

Three further tests of the Na^+-gradient hypothesis are applied. These, like one used earlier, support the hypothesis, which is therefore considered to be established. The findings with the three new tests are as follows. (1) Two Na ions enter the cells in concert with one glycine, as expected from the previously reported kinetic dependence of glycine entry on $(Na^+)^2$. (2) A system with high but equal concentrations of Na^+ inside and out, which does not pump glycine due to the absence of a Na^+ gradient, can be caused to pump glycine (out) by a Donnan effect. In the presence of the Donnan electrical potential there is a Na^+-electrochemical gradient even though there is no Na^+-concentration gradient. (3) No correlation is found between the concentration of cell nucleotide polyphosphate(s) (ATP) and glycine-pump activity.

Total glycine entry into pigeon red cells can be considered to consist of two components: entry by a sodium-dependent route which obeys Michaelis-Menten kinetics with respect to both glycine and $(Na^+)^2$, and a small diffusionlike route. The Na^+ dependence implies the existence of a complex containing both Na^+ and glycine at some stage in the entry process. Part of the glycine exit from Na^+-enriched cells is also Na^+ dependent (Vidaver, 1964a).

Pigeon red cells, like mammalian red cells, can be hemolyzed and restored (made again selectively permeable). The cation and glycine concentrations in the restored cells are largely determined by the cation and glycine concentrations in the lysing and restoring solutions. Such preparations can pump glycine, but only if a sodium gradient exists (Vidaver, 1964b) These experiments with lysed and restored cells supported Christensen's hypothesis that the difference in Na^+ concentration between the cell interior and medium furnishes the energy for amino acid–active transport (Christensen *et al.*, 1952; Riggs *et al.*, 1958).

Several predictions made from the hypothesis could be used to test it. If energy comes from the Na^+ gradient, the energy in it must be expended; that is, Na^+ must move down its gradient, and some of this movement must be coupled to glycine movement against the glycine gradient. From the kinetic dependence of glycine entry on $(Na^+)^2$, two Na ions would be expected to enter the cell for every glycine entering by the Na^+ dependent route. The test of this prediction will be referred to as the "stochiometry test."

It had been found that replacement of Cl^- of the medium by mucate produced a Donnan effect with its accompanying electrical potential (Vidaver, 1964c). Hemolyzed and restored cells with equal internal and external concentrations of both Na^+ and glycine

* The work described in this paper was supported by research grants to Professor F. Haurowitz from the National Science Foundation (NSF G16345) and the U. S. Public Health Service (NIH RG1852), and by contracts of Indiana University with the Office of Naval Research (Nonr-3104[00]) and the Atomic Energy Commission (AEC AT[11-1]-209).

TABLE I
INCREASE IN Na^+ ENTRY WITH INCREASE IN GLYCINE ENTRY[a]

Cl_o^- (mM)	Glycine (mM)	Na^+-dependent Glycine Entry (μmoles/ml pellet H_2O in 21 min)	Increment in Glycine Entry	Na^+ Entry (μmoles/ml pellet H_2O in 21 min)	Increment in Na^+ Entry	Ratio of Na^+-Entry Increment to Glycine-Entry Increment
3	0.3_1	0.05_1	0.19	1.0_9	0.35	1.9
	3.0	0.23_8		1.4_4		
7	0.3_1	0.06_8	0.27	1.1_3	0.59	2.2
	3.0	0.34_1		1.7_3		
30	0.3_1	0.15_2	0.49	2.1_5	0.76	1.5
	3.0	0.64_5		2.8_9		
83	0.3_1	0.27_5	0.54	2.2_3	0.98	1.8
	3.0	0.81_8		3.2_1		

[a] Cell Cl^- was exchanged for methanesulfonate by washing intact cells with K^+ methanesulfonate, resuspending with one volume of K^+ methanesulfonate and incubating 5 minutes at 39°, centrifuging, resuspending with 5.2 volumes K^+ methanesulfonate, and incubating 10 minutes at 39°. Cell Cl^- was thereby reduced to 4 μmoles/ml cell H_2O. Pellets of these cells were suspended with 2.00 ml of the appropriate medium. All media contained 43 mM ^{22}Na-labeled Na^+, Cl^- plus methanesulfonate totaling 142 mM, and [^{14}C]glycine. Samples were incubated 21 minutes at 39°. Samples were processed, and incubations and calculations were performed as described previously (Vidaver, 1964a) except for one modification; after draining the pellets, the insides of the tubes were swabbed. The Na^+-dependent glycine entry is total glycine entry minus the Na^+-independent component calculated from entry from Na^+-free medium. The "increment in glycine entry" is Na^+-dependent glycine entry from 3.0 mM $glycine_o$ medium minus that from 0.3_1 mM $glycine_o$. "Increment in Na^+ entry" is Na^+ entry from 3.0 mM $glycine_o$ medium minus that from 0.3_1 mM $glycine_o$.

showed equal glycine entry and exit rates, i.e., they did not pump glycine (Vidaver, 1964b). If a Donnan electrical potential were imposed on such a nonpumping system by a nonpenetrating anion in the medium, there would be an electrochemical Na^+ gradient, with Na^+ inside at a higher potential than that outside, even though Na^+ concentrations would still be equal inside and out. The cells should then pump glycine out. The test of this prediction will be referred to as the "induced-pump test."

Finally, the ability of cells to pump glycine should not depend on their content of ATP (or other nucleotide polyphosphates). The test of this prediction will be referred to as the "ATP test."

The above predictions were tested and the results found agreed with the predictions.

MATERIALS AND METHODS

Procedures for preparation of cells, lysis and restoration, incubation, and [^{14}C]glycine counting; analysis for Na^+, Cl^-, glycine, and extracellular space; and the preparation of toluene-2,4-disulfonates were generally as in previous reports (Vidaver, 1964a,b,c). Any modifications used are given below or in the legends of the tables or figures.

For determination of ^{22}Na in the presence of ^{14}C, 0.40_0-ml samples of picric acid extracts were spread over the bottoms of ringed stainless steel planchets, 2.5 × 0.8 cm. (Atomic Accessories SP-132) together with 2 drops of 1.4% sodium dodecylsulfate (Duponol) and air dried. These were counted through an aluminum window (19–21 mg/cm²; made by cementing three layers of 1-mil aluminum foil over a Nuclear Chicago "micromil window" ring). This screened out *ca.* 65% of the ^{22}Na counts but 99.8% of the ^{14}C counts. Samples were plated in duplicate and the radioactivity of each planchet was counted for a total of 3200 counts.

For determination of [^{14}C]glycine in samples containing ^{22}Na, the thick-sample plating procedure (Vidaver, 1964a), in which ^{14}C was coprecipitated with carrier glycine by acetone, was modified by the inclusion of *ca.* 0.15 M sodium picrate and *ca.* 0.02 M picric acid in the acetone used for the first acetone precipitation. The ^{22}Na was removed as sodium picrate by the acetone washes.

Nucleotide polyphosphate (ATP) is the charcoal-adsorbable acid-labile phosphate which is labile to potato apyrase. Slightly modified versions were used of the charcoal-adsorbtion procedure of Crane and Lipman (1953) and the phosphate analysis method of Martin and Doty (1949). Cell pellets were promptly extracted with 5.00 ml cold trichloroacetic acid, final concentration, 6%. (Pellets could not be stored frozen since ATP was lost on freezing and thawing.) Aliquots of the cold extracts were mixed with 1 ml 10% (w/v) acid-washed Norit A decolorizing charcoal (final volume, *ca.* 6 ml), thoroughly stirred three times, and centrifuged. The pellets were washed four times with 3 ml deionized H_2O; this included rinsing the inside and outside of the mouth of the inverted tube and stirring rod. All of these operations were done in the cold. The charcoal pellets were suspended with 3.00 ml 0.1 N HCl, heated 8 minutes in a boiling-water bath, cooled, and centrifuged. The supernatants were mixed with 0.4 ml of 5.8 N NaOH, 0.8 ml of the acid molybdate reagent of Martin and Doty (1949), and 2.5 ml of 1:1 isoamyl alcohol–benzene. They were thoroughly mixed three times with a vibrator. To 2.00 ml of the upper layer was added 0.1 ml $SnCl_2$ reagent (Martin and Doty, 1949) made with alcoholic H_2SO_4 instead of aqueous H_2SO_4. The color was read in 2 ml Coleman tubes at 660 mμ. The extinction produced by 0.02 μmoles ATP in the original aliquot of trichloroacetic acid extract was 0.10–0.11. The apyrase-labile components were determined by mixing 0.3–0.5 g frozen and thawed pellets with 0.20 ml potato apyrase solution and 3.8 ml of a solution containing 139 mM KCl, 4.5 mM KH_2PO_4, 4.5 mM K_2HPO_4, 3.8 mM $MgSO_4$, and 1.9 mM $CaCl_2$. After being incubated for 30 minutes at 35–37°, these were chilled, deproteinized with trichloroacetic acid (final concentration, 6%; final volume, 5 ml), and analyzed for charcoal-adsorbable acid-labile phosphate as described above. The value for P_i per sample thus obtained was subtracted from the value of P_i per sample from the

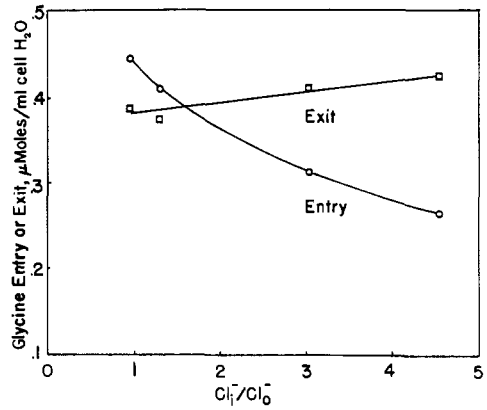

FIG. 1.—Total (i.e., Na^+-dependent plus Na^+-independent) glycine entry and exit plotted against Cl_i^-/Cl_o^-. (The Cl_i^-/Cl_o^- ratio reflects the Donnan electrical potential produced by toluenedisulfonate$_o$.) Cells were lysed and restored with K^+-free solutions. The lysing solutions contained [^{14}C]- or [^{12}C]glycine. Glycine exit and entry were measured to and from pairs of samples. One member of each pair had ^{14}C inside and ^{12}C outside, the other, ^{12}C inside and ^{14}C outside. Otherwise the cell composition and incubation media were identical for both members of each pair. The entry rates given include a "re-exit" correction. A fraction of the internal glycine leaves the cell during incubation. The re-exit correction used was 0.5 × this fraction, as estimated from ^{14}C loss from the internally labeled member of the pair, times the measured ^{14}C entry into the other member. The corrected cpm entering, divided by cpm/μmole glycine$_o$, is glycine entry. The lysed and restored cells were incubated 10 minutes at 39° in KRPG media, 125 mM in Na^+ and 1.5 mM in glycine, with varying proportions of Cl_o^- replaced by toluenedisulfonate (plus sucrose). (Toluenedisulfonate was used instead of mucate [Vidaver, 1964c] to avoid precipitation of calcium mucate.) The average cell Na^+ was 128 mM (it varied a few per cent with toluenedisulfonate and incubation). Glycine$_i$ was 1.5 mM. Cell Cl^- varied from 134 to 104 mM over the toluenedisulfonate range of zero to 62 mM, while Cl_o^- ranged from 141 to 23 mM over the same toluenedisulfonate range. Some Cl^- left the cells during incubation (the maximum loss, 14%, occurred at the lowest Cl_o^-); the Cl_i^- and Cl_o^- values used are the averages of incubated and unincubated sample values. The preparative, incubation, and analytical procedures used are indicated under Materials and Methods.

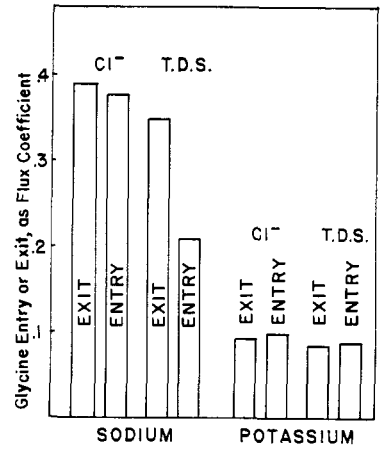

FIG. 2.—In this experiment, the action of the Donnan potential on glycine entry and exit when both cells and media were high in Na^+ ("sodium"; as for Fig. 1) is compared with its action when cells and media had (almost) no Na^+ ("potassium"). The procedures were as described in the legend of Fig. 1 except that the "lysing" and "restoring" solutions for the Na^+-poor samples contained only K^+, and only one toluenedisulfonate (T.D.S.) concentration was used. Incubation was for 11 minutes at 39°. The cells obtained were (except for Na_i^+ and K_i^+ of the Na^+-poor cells) like those in the experiment of Fig. 1. The results are given as flux coefficients. These are: cpm moving during incubation, divided by (for exit) average cpm of incubated and unincubated cells, or (for entry), cpm of a volume of medium equal to the cell H_2O in the pellet. A re-exit correction was used as in Fig. 1.

corresponding cell sample not treated with apyrase.[1] This was multiplied by 0.5, (ATP/acid-labile P_i), and by 0.61 (an empirical "ATP recovery factor"). This apyrase-labile component was the ATP of a cell pellet. Most of the charcoal-adsorbable acid-labile phosphate was labile to apyrase.

The potato-apyrase solution used was prepared as described by Lee and Eiler (1951). The preparation was neutralized with Tris base and stored frozen until use. A 1:30 dilution of this preparation, in the salts and buffer mixture given above, split all of the terminal and 67% of the subterminal phosphate from 0.15 mM ATP in 10 minutes at 38°.

RESULTS

To determine the correlation between Na^+ entry and glycine entry (the stoichiometry test), the increment in ^{22}Na entry produced by changing glycine$_o$ from 0.3 mM to 3.0 mM was compared with the increment in the Na^+-dependent component of [^{14}C]glycine entry produced by this same change in glycine$_o$. If the entry of two Na ions is coupled to the entry of one glycine, the ratio, increment in Na^+ entry/increment in glycine entry, should be 2, even though the numerical value of the increment in glycine entry is varied. Glycine entry was varied by varying Cl_o^- (Vidaver, 1964c). The data of Table I show that the ratio of increments was *ca.* 2 over a 3-fold range of the glycine-entry increment.

The entry-increment ratios listed cannot be highly accurate since the Na^+-entry values were not corrected for re-exit of ^{22}Na during the course of the incubation. No reasonable assumption could be made about the rate of mixing of newly entered ^{22}Na with Na^+ already there. No glycine-re-exit corrections were applied here either, although for glycine it is at least plausible to suppose mixing is rapid since internal glycine appears to be "free" (Vidaver, 1964a).

The major anion in the medium in this experiment was methanesulfonate. Cell Cl^- had been exchanged for methanesulfonate before incubation with ^{22}Na and [^{14}C]glycine was begun.

The results of the induced-pump test are shown in Figures 1 and 2. In the experiment shown in Figure 1, glycine and Na^+ (chiefly as NaCl) were introduced into the cells by hemolysis and restoration (Vidaver, 1964b), and the cells were incubated in media containing glycine and Na^+ at the same concentrations as those inside the cells. When the chief anion in the medium was Cl^-, glycine entry and exit were substantially equal. Replacement of Cl_o^- by the nonpenetrating anion, toluene-2,4-disulfonate, was used to produce the Donnan effect. Figure 1 shows that as the Donnan-associated electrical potential increased in a direction inhibiting entry and promoting exit of cations, glycine entry decreased while glycine exit did

[1] Abbreviations used in this work: P_i, inorganic phosphate; KRPG, the slightly modified Krebs Ringer phosphate glucose solution used in previous work (Vidaver, 1964a). The subscript "*o*" or "*i*" after a symbol for or name of a substance means the substance represented is present in the medium or the cell, respectively (except in the case of P_i).

TABLE II

GLYCINE PUMP CAPACITIES AND ATP CONTENTS OF INTACT CELLS, LYSED AND RESTORED CELLS, AND CELLS LYSED IN THE PRESENCE OF APYRASE AND THEN RESTORED[a]

Cell Type	Cell H_2O (ml/ pellet)	Na_i^+ (μmoles/ ml cell H_2O)	Na_o^+ (mM)	$Glycine_i$ (μmoles/ ml cell H_2O)	$Glycine_o$ (mM)	Total Glycine Entry (from ^{14}C data) (μmoles/ pellet in 10 min)	Glycine Efflux Coefficient for 10 min	Virtual Net Glycine Entry (μmoles/ pellet in 10 min)	ATP (μmoles/ pellet)
Intact, before incubation	0.34	16.9	146	3.50					1.78
									1.72
					0.82	0.179	0.070	0.161	
Intact, after incubation	0.33	19.3	146	3.95					1.80
									1.82
Avg.	0.33							Avg.	1.78
Lysed and restored before incubation	0.39	9.4	136	0.55					0.116
									0.116
					0.76	0.119	0.069	0.097	
Lysed and restored, after incubation	0.39	22.3	134	0.83					0.122
									0.125
Avg.	0.39							Avg.	0.120
Lysed and restored with apyrase, before incubation	0.38	7.1	136	0.56					0.069
									0.082
					0.84	0.117	0.071	0.096	
Lysed and restored with apyrase, after incubation	0.37	22.7	134	0.82					0.074
									0.083
Avg.	0.38							Avg.	0.077

[a] Intact cells were "loaded" with either [^{14}C]- or [^{12}C]glycine by incubating as 50% (w/v) suspensions in K-KRPG (this is KRPG with all Na^+ replaced by K^+) containing 1.1 mM glycine (either 1 μc/μmole, or unlabeled) for 30 minutes at 39°. After being centrifuged and washed with K-KRPG (9500 rpm in a Servall SS1 head, 10 minutes, 5–7°), aliquots of both ^{14}C- and ^{12}C-loaded cells were stored until use (intact cells), lysed with lysing solution containing 0.32 ml apyrase per 10 ml and restored, or lysed without apyrase and restored. Lysis and restoration were as described before (Vidaver, 1964b) except that a 9-minute exposure to 0° was inserted before the "annealing" step. The lysing and restoring solutions contained only K^+ salts. The annealed cell suspensions were diluted with three volumes of K-KRPG and centrifuged 10 minutes at 4° at 2600 rpm (radius to midpoint of tube, 12 cm). These pellets and the intact-cell pellets were suspended in Na-KRPG media (KRPG with all K^+ replaced by Na^+) containing [^{12}C]- or [^{14}C]glycine. Cells loaded with ^{14}C were put in ^{12}C media and vice versa. (*Note:* lysed and restored pellets were much more loosely packed than intact cell pellets, thus Na_o^+ was higher for intact-cell suspensions.) Two 3.00-ml aliquots of each of the six suspensions were incubated 10 minutes at 39°. Corresponding unincubated controls were centrifuged immediately. Suspensions with ^{14}C in the medium were diluted with 5.0 ml K-KRPG immediately before centrifuging; those with ^{14}C inside were centrifuged without dilution. Pellets were either deproteinized immediately with trichloroacetic acid (for analysis for charcoal-adsorbable acid-labile phosphate and radioactivity) or stored frozen (for analysis for apyrase-resistant, charcoal-adsorbable, acid-labile phosphate, radioactivity, Na^+, and glycine). Sodium and glycine were measured in picric acid extracts of both pellets and media. Radioactivity was determined with thick-sample plates (Vidaver, 1964a). Steps in the procedures not described here or in the text were as described previously (Vidaver, 1964a,b). Extracellular space (not shown) was calculated by comparison of Na^+ per pellet values of diluted and undiluted suspensions. It has been assumed throughout that cells containing [^{14}C]-glycine and cells containing [^{12}C]glycine are chemically identical. The units used for glycine exit and entry, which differ from each other, were chosen to facilitate the comparison of intact cells with lysed and restored ones, and the total glycine entry with virtual net entry. Cell H_2O per pellet values are listed to allow calculation of exit and entry on other bases. For calculation of glycine entry into lysed and restored cells, a value for $glycine_o$ of 0.80 mM was used. Glycine efflux coefficient is: cpm moving into the medium from [^{14}C]glycine-containing cells during incubation, divided by the average cpm of cells before and after incubation. Glycine entry is: cpm entering cells divided by cpm/μmole $glycine_o$. "Virtual net glycine entry" is: (glycine entry per pellet) − (glycine efflux coefficient × $glycine_o$ × ml cell H_2O per pellet). It is assumed that the glycine-efflux coefficient of intact cells is essentially independent of $glycine_i$ under the conditions of the experiment. For purposes of assessing pump capacity this is a reasonable assumption (Vidaver, 1964a). For lysed and restored cells, it is assumed that the efflux coefficient used (0.070) would not be significantly different if $glycine_i$ were equal to $glycine_o$ instead of only similar.

not. Thus glycine exit became greater than entry, though internal and external glycine concentrations were equal; i.e., glycine was pumped out.

Figure 2 shows that this effect occurred only in the presence of Na^+. That is, it is an effect mediated by a Na^+-dependent glycine-transfer route. It had already been concluded (Vidaver, 1964a) that a Na^+-dependent route was responsible for glycine accumulation by intact pigeon red cells.

The results of the ATP tests are shown in Table II. Lysed and restored cells contained very little ATP. The reduction in ATP content was much greater than that expected from the 6-fold dilution of contents occurring during lysis and restoration. Cells lysed with a lysing solution containing potato apyrase and then restored contained somewhat less ATP than cells lysed and restored in the absence of apyrase.

In Table II, the "virtual net glycine entries" into intact cells, into cells lysed in the presence of apyrase and then restored, and into cells lysed and restored without apyrase are compared. Virtual net glycine entry is the total (i.e., Na^+-independent plus Na^+-dependent)

glycine entry from high-Na^+ medium minus the total glycine exit that would have occurred if the internal glycine concentration had been equal to the external. This is used as the quantitative measure of the capacity of the cells to actively pump glycine. Virtual net glycine entries and ATP contents are given as μmoles per pellet rather than μmoles/ml cell H_2O, since lysed and restored cells have more H_2O per cell than intact ones (Vidaver, 1964b). Pellets of lysed and restored cells and pellets of intact cells were prepared from the same number of cells. Comparisons are therefore made on the basis of equal cell numbers or, initially, equal quantities of pump mechanism. For ATP, concentration may be more important than quantity per pellet (see below).

In this experiment, the virtual net glycine entry values for lysed and restored cells with and without apyrase were equal to each other and were 60% of the value for intact cells. Cells lysed and restored without apyrase had 6.7% as much ATP as intact cells on a per pellet basis or 5.7% as much on a concentration basis. Cells lysed and restored with apyrase had 67% of the ATP of cells lysed and restored without it, and 4.3% as much ATP as intact cells (per pellet) or 3.9% as much on a concentration basis. Thus there did not seem to be any significant correlation between the ability of cells to actively pump glycine and their ATP contents.

Discussion

The hypothesis, that the Na^+ gradient furnishes the energy for glycine-active transport, requires that the entry of glycine into the cell against its concentration gradient be coupled to Na^+ entry down its concentration gradient. Since Cl_i^-/Cl_o^- for intact cells in KRPG is not far from one, (Vidaver, 1964c), the electrical potential is small, and the electrochemical Na^+ gradient is primarily due to the Na^+-concentration gradient.

Glycine entry obeys Michaelis-Menten kinetics with respect to both glycine and $(Na^+)^2$. Therefore the number of sodium ions entering with each glycine would be expected to be constant. Otherwise a change in mechanism and kinetic dependence on Na^+ should occur with changes in glycine-entry rate. Moreover, the equation implies that there is a major rate-limiting step involving a complex containing two sodium ions and one glycine. It might be expected that the slowest step in the overall glycine-entry process would be the actual transfer of material across the cell membrane, and hence that this step is the step involving the complex containing two sodium ions and one glycine. It would then be expected that an increase in glycine entry would be accompanied by an increase in Na^+ entry, and that the increase in Na^+ entry would be proportional to the increase in glycine entry, with the ratio of increases 2:1. The data of Table I fit these expectations reasonably well.

Determination of the ratio, Na^+ entry increment/glycine entry increment, at several glycine entry increments, was feasible because lack of Cl_o^- restricted basal Na^+ entry. Less satisfactory stoichiometry was obtained in attempts to measure the increment ratio at several glycine increment values produced by varying glycine over a range, or by varying Na_o^+. Technical difficulties arose directly or indirectly from the large size of the basal Na^+ entry relative to the Na^+-entry increments being measured.

As discussed in the introduction, if a system is initially not pumping glycine because internal and external Na^+ are equal and therefore no energy is available, the imposition of an electrical field should induce pumping. With the field present, an electrochemical Na^+ gradient would exist even though internal and external Na^+ concentrations are equal. With the field direction produced by the nonpenetrating anion in the medium, glycine should be pumped out.

The outpumping found was due chiefly to a reduction of glycine entry. It had been found that Cl_o^- was required for glycine entry into intact red cells in the absence of an electric field (Vidaver, 1964c). Much of the induced pumping by lysed and restored cells might therefore be directly due to the Cl^--concentration difference maintained between cells and medium by the Donnan effect. However, the energy for pumping cannot come from this Cl^- difference. Energy cannot be stored in a Cl^- gradient because the cells are freely permeable to Cl^-, and the Cl^- therefore is at its equilibrium distribution. Usable energy can be stored in an ion-electrochemical gradient only when the ions involved cross the membrane slowly. The main ions meeting this requirement are Na^+ and K^+. Since entry of glycine into intact cells is not sensitive to K^+ (Vidaver, 1964a), K^+ is unlikely to be involved. Moreover, when cells with high K_i^+ are incubated in a high Na^+ medium, the direction of the K^+-electrochemical gradient (largely due to the K^+-concentration gradient) is from inside to outside. Such cells pump glycine in. In the present case, the induced electrochemical gradient for cations is also directed from inside to outside. If K^+ were the responsible ion, these cells should also pump glycine in. Instead, they pump it out. If the energy comes from an electrochemical gradient produced by the field, it is probably the Na^+ gradient, with Cl^- acting only as a mediator.

The results of the ATP test (Table II) reduced the likelihood that ATP is the immediate energy source for glycine transport and thus indirectly, by weakening the main alternative, supported the Na^+ gradient hypothesis. (ATP is presumably the energy source for the Na^+ gradient, and thus indirectly for the glycine pump.)

There did not appear to be a correlation between cell ATP and glycine pump activity. Although the pump activity of the lysed and restored cells was only 60% of that of intact cells, much of this loss is probably due to damage by the lysis and restoration process. The loss of pump activity is slight compared to the loss of ATP on lysis and restoration. The further reduction of ATP due to the inclusion of apyrase was not accompanied by further reduction in pump activity. This drop in ATP, while modest, was quite significant. A percentage drop in "pump activity" a third the size of the ATP drop would have been detected.

The effective ATP in cells lysed in the presence of apyrase may be much lower than the measured amount. If the apyrase were freely distributed between cell and lysate H_2O during lysis, it would be present in the cell in the same concentration as that used for the apyrase assay (see Materials and Methods). It acted for 17 minutes at 37–39°. Under the assay conditions, this much apyrase destroyed 83% of the ATP in 10 minutes at 38°. The relative ineffectiveness of the introduced apyrase may be illusory. Pigeon red cells probably have ATP in the nucleus, as do chicken red cells (Osawa *et al.*, 1957). If apyrase did not enter the nucleus during lysis, the residual ATP might have been entirely nuclear and not available to the glycine pump.

Discussion has been in terms of ATP, but other nucleoside di- and triphosphates are, in effect, either included under ATP or else largely absent. The apyrase treatment used in the ATP-assay procedure

destroyed 85–91% of the total charcoal-adsorbable acid-labile phosphate of lysed and restored cells, and 98.5% of that of intact cells. Nucleoside pyrophosphates of bases other than adenine must have been nearly absent, hydrolyzed by apyrase, or converted to ATP by the action of cell transphosphorylases.

If the pump activity had shown any correlation with cell ATP, the Na^+-gradient hypothesis would have been greatly weakened; but the lack of correlation observed does not strengthen it correspondingly. A pump with ATP as energy source might show no such correlation if the ATP were generated in a small compartment of the cell which neither lost much ATP nor admitted apyrase during lysis. Also, the pump might be saturated at very low concentrations of ATP. For such reasons, experiments of this type cannot rule out the action of ATP.

One test of the Na^+ gradient hypothesis had been applied earlier (Vidaver, 1964b). It was shown that glycine pumping required a Na^+ gradient, and that the direction of the Na^+ gradient determined the direction in which glycine was pumped. Including this earlier test, a total of four independent tests have been applied. The results of each conform to the predictions from the hypothesis. Each test was as capable of weakening the hypothesis as strengthening it. Two tests, the induced-pump test and the test reported in the earlier paper, were capable of flatly disproving it. These four therefore were all fair tests. Although no one test by itself is conclusive, taken together they appear to establish the hypothesis as correct.

ACKNOWLEDGMENTS

The author wishes to thank Professor Felix Haurowitz for his advice and support throughout the course of this work. He also wishes to thank Mr. Roger Stickney and Mr. Michael Bourn for technical assistance.

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Differences in the Metabolism of *N*-Hydroxy-*N*-2-fluorenylacetamide in Male and Female Rats*

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Received January 13, 1964

Studies on metabolism of carbon-14-labeled *N*-hydroxy-*N*-2-fluorenylacetamide (N-OH-2-FAA) were performed in Fischer rats to elucidate the mechanism of action of this carcinogenic chemical. Adult male rats excreted less of a dose in the urine than female rats. The urine of male rats contained more sulfate conjugates and less of the glucuronic acid conjugates than that of female rats. The ether-soluble metabolites after hydrolysis of the glucosiduronic acids contained 7-, 5-, and 3-hydroxy-FAA in addition to N-OH-FAA. In the urine of female rats there was considerably more N-OH-FAA whereas in males there was more 5- and 7-hydroxy-FAA. The sulfuric acid ester fraction contained mostly 7-hydroxy-FAA and 7-amino-2-fluorenol. More radioactivity was bound to liver proteins in male than in female rats but the kidney proteins of males and females contained approximately equal amounts of activity. Thus there was a sex difference in the metabolism of the carcinogen *N*-hydroxy-FAA in Fischer-strain rats.

There is a considerable body of evidence that in addition to species and strain effects, there may be sex-linked differences in the metabolism of various drugs (Axelrod, 1956; Quinn *et al.*, 1958). Thus male rats acetylate sulfanilamide more extensively than do females (Franz and Lata, 1957). On the other hand, homogenates or liver-cell fractions from female rats metabolized steroid hormones at a faster rate than did those from males (Yates *et al.*, 1958; Leybold and Staudinger, 1959).

In the case of the carcinogenic aromatic amine derivative *N*-2-fluorenylacetamide (FAA),[1] we have shown that there is only a limited influence of sex on the metabolism of a single dose of this compound. There was a tendency for greater urinary levels of hydroxylated metabolites conjugated with sulfuric acid in the mature male rats while mature females excreted more glucuronides (Weisburger and Weisburger, 1963a). In this connection it should be mentioned that there is a significant difference in the response of male and female rats to the carcinogenic action of FAA, the livers of males being appreciably more susceptible. Moreover, hypophysectomy, adrenalectomy, gonadectomy, and similar modification of the hormonal milieu

* Presented in part at the 147th National Meeting of the American Chemical Society, Philadelphia, April 1964.

[1] Abbreviations used in this work: FAA or 2-FAA, *N*-2-fluorenylacetamide; N-OH-FAA, *N*-hydroxy-*N*-2-fluorenylacetamide (*N*-2-fluorenylacetohydroxamic acid); 3-OH-FAA, the 3-hydroxy derivative, etc.; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2-(5-phenyloxazolyl)benzene; DEAE, diethylaminoethyl-; 7-OH-2-FA, 7-hydroxy-2-fluorenamine.