Biochimica et Biophysica Acta, 323 (1973) 441–455

BBA 76471

THE EFFECT OF GRAMICIDIN ON SODIUM-DEPENDENT ACCUMULATION OF GLYCINE BY PIGEON RED CELLS: A TEST OF THE CATION GRADIENT HYPOTHESIS*

PHILIP M. TERRY and GEORGE A. VIDAVER

Department of Chemistry, University of Nebraska, Lincoln, Nebr. (U.S.A.)

(Received July 23rd, 1973)

SUMMARY

1. At 3 μg/g cells or more, gramicidin equilibrates cell and medium Na^+ and K^+ within 3 min at 39 °C. At equilibrium, cell and medium cation concentrations are approximately equal.

2. Gramicidin virtually abolishes Na^+-dependent glycine accumulation. The slight residual accumulation may be caused by small gramicidin-induced cell volume changes.

3. The inhibition of glycine accumulation by gramicidin could not be accounted for by inactivation of the glycine carrier, increased Na^+-independent loss (leak) of glycine from cells, or depletion of ATP or other nucleoside polyphosphates. Competition between the Na^+–K^+-pump and glycine transport for a common high-energy intermediate is not indicated since strophanthin k (gramicidin absent) had only a minimal effect on glycine accumulation.

4. Other possible energy sources besides cation gradients were considered. Under the conditions used, there were negligible contributions from counter-transport *via* the ASC route (Eavenson, E. and Christensen, H. N. (1967) *J. Biol. Chem.* 242, 5386–5396), a Cl^- gradient or a H^+ gradient.

5. Some problems in evaluating the adequacy of cation gradients as sole energy sources are discussed.

6. Virtually all of the energy for glycine accumulation by pigeon red cells appears to come from cation gradients.

INTRODUCTION

In many systems, active transport of organic solutes depends on Na^+ in the external medium. In several of these systems, transport of organic solutes and Na^+ (and possibly K^+) are coupled, so that energy for active organic solute transport comes from the movement of Na^+ (and possibly K^+) down their electrochemical gradients[2].

* A preliminary report of some of this work has appeared[1].

** We report gramicidin levels in μg/g cells. We do not know whether the gramicidin/g of cells or the gramicidin/ml medium is the appropriate measure of gramicidin level. Cell concentrations were the same in all experiments, so both measurements are equivalent here.

Several laboratories working with several different systems recently reported Na^+-dependent amino acid or sugar accumulation in excess of that accounted for by the apparent cation gradients[3–13]. These observations imply that some energy source acts besides the cation gradients. The energy obtainable from cation gradients is calculated with the assumption that internal cations are not compartmented or bound. However, the actual cation electrochemical activity gradients are inadequately known (see below).

Both intact[14,15] and hemolysed and restored[13,15,17] pigeon red cells transport glycine by a Na^+-dependent route. The Na^+ gradient, according to its direction, drives either glycine accumulation or expulsion[13–17]. Integration of rate equations[17], which embody no energy source other than the Na^+ gradient, approximately gives the glycine accumulation and expulsion observed with hemolysed and restored pigeon red cells[13]. However, discrepancies exist. Accumulation is a little greater and expulsion a little less than calculated. These discrepancies might be due to some additional energy source or to accumulated errors in the simplifying assumptions and approximations used.

We wished to devise a test to determine if all of the energy for glycine transport comes from cation gradients; preferably one also applicable to other systems. Since intact cells seemed more likely than the hemolysed and restored preparation to have (a) an additional energy source or (b) cation compartmentation or binding, the intact pigeon red cell system was tested.

Gramicidin is an ionophore showing little discrimination between Na^+ and K^+; K^+ is favored by only 2–6-fold[18–20]. Gramicidin can therefore abolish any Na^+ or K^+ electrochemical activity gradient regardless of cation compartmentation, cation binding, or membrane potentials. If no energy source exists for glycine active transport except the Na^+ or K^+ gradients, gramicidin should abolish glycine accumulation. Gramicidin virtually abolished glycine accumulation by intact pigeon red cells.

To find the right conditions for performing this test, we examined the effects of gramicidin on Na^+ and K^+ movement in pigeon red cells. We also examined some possible effects of gramicidin, other than the dissipation of the cation gradients, which might have inhibited glycine accumulation.

MATERIALS AND METHODS

Heparin sodium, Gramicidin D (a mixture of Gramicidins A, B and C), ATP (99–100% as the disodium salt), Hexokinase Type F-300, and Sigma kit No. 366-UV (for enzymatic determination of nucleoside triphosphates) were all obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. AMP was obtained from Calbiochem., San Diego, Calif., U.S.A. [1-^{14}C]Glycine, 1–10 Ci/mole was obtained from New England Nuclear, Boston, Mass., U.S.A. Other chemicals used were reagent grade from standard commercial sources and were used without further purification.

Pigeon red cells were prepared and incubated in modified Krebs–Ringer phosphate as previously described[14]. Cells and media were extracted with picric acid and the Na^+ and K^+ in the extracts measured with a Baird-Atomic KY-3 flame photometer using Li^+ as internal standard.

Glycine was determined for us with a Spinco amino acid analyser by Professor Paul J. Mattern by a standard procedure with norleucine as internal standard. We

were informed that reliability was better than 3%. Agreement between measured and calculated glycine concentrations in the media was better than 3% in all but one of 38 samples.

Glycine accumulation capacity was determined by incubating aliquots of cells in media with different glycine concentrations such that cells accumulated glycine from some media and lost it to others. The glycine accumulation ratio the cells can just maintain is the initial cell glycine concentration divided by the external glycine concentration where cells neither gained nor lost glycine. This external glycine concentration is the x coordinate of that point on the plot of cell glycine concentration after incubation *vs* external glycine concentration whose y coordinate is the initial cell glycine concentration. Such points are seen in Fig. 2 as the intersections of the solid slant lines with the horizontal dashed line representing the initial cell glycine concentration.

The β *plus* γ phosphate groups of all nucleoside polyphosphates were determined as charcoal-adsorbable acid-labile phosphate as described before[15] except that potato apyrase was not used and phosphate was determined by the procedure of Gomori[21].

Nucleoside triphosphates and ATP were determined with the Sigma kit[22]. Treatment of samples was identical to that in the nucleoside polyphosphate determination up to the trichloroacetic acid deproteinization step where 3.00 ml of 0.364 M $HClO_4$ replaced trichloroacetic acid. The pellets were immediately suspended with stirring rods and resuspended several more times in the course of 10 min at 0 °C. The samples were centrifuged, 2.00-ml aliquots of supernatant were neutralized with $KHCO_3$, the $KClO_4$ was centrifuged off and the supernatant from each sample split into two aliquots. One aliquot was incubated 10 min, 25 °C with 2 units/ml hexokinase in 0.06 M, Tris buffer, pH 8.1 (final concentrations). The reaction was stopped with cold trichloroacetic acid final concentration 5% (w/v). Trichloroacetic acid was added to the unincubated aliquot and all samples centrifuged. After holding overnight at 0 °C all samples were analysed for nucleoside triphosphate with the Sigma kit[22]. Hexokinase is fairly specific for ATP among the naturally occurring nucleoside triphosphates[23], and the hexokinase treatment used sufficed to just consume ATP but leave the next best substrate ITP, virtually untouched. The Sigma kit method determines total nucleoside triphosphates. The hexokinase-labile portion is ATP.

RESULTS AND DISCUSSION

Effects of gramicidin on cell Na^+ and K^+

As illustrated by Fig. 1, with 3 μg gramicidin/g wet weight of cells, a constant level of Na^+ in the cells is reached within 3 min at 39 °C. The data in Table I shows that at or above 3 μg/g of cells both Na^+ and K^+ permeation are very rapid and by 3 min the Na^+ and K^+ in the cells are independent of the gramicidin level (compare Na_i^+ and K_i^+ at various gramicidin levels). The data in Table II show that, in the absence of sucrose in the medium, Na_i^+ and K_i^+ approach Na_0^+ and K_0^+, respectively, quite closely over a wide range of Na_0^+ and K_0^+ values, except possibly at very low Na_0^+ or K_0^+, where Na_i^+/Na_0^+ and K_i^+/K_0^+ may exceed unity. Table III shows the effect of 31 mM sucrose on Na_i^+ and K_i^+ after a 3-min incubation with

TABLE I

EFFECTS OF VARIOUS LEVELS OF GRAMICIDIN ON CELL Na^+ AND K^+

10 ml of cold cell suspension, 2.5% (w/v) in modified[14] Krebs–Ringer phosphate medium containing the listed Na^+ and K^+ concentrations, were dumped into a tube containing 0.10 ml of a gramicidin solution in ethyleneglycol–ethanol (9:1, v/v). Each sample was immediately mixed by pouring back and forth between tubes several times and then placed in the 39 °C bath at zero time. After 3 min, each tube was chilled for 3 min and centrifuged. Pellets and supernatants were diluted with water as necessary to keep [picrate]·$[K^+]$ below the solubility product of potassium picrate and deproteinized at room temperature with picric acid (final concn, half saturated). Sodium and potassium in the extracts were determined by flame photometry. Cell Na^+ and K^+ contents were calculated using the wet weights of these pellets and ml cell water/ml pellet obtained in other experiments. Pellet water is pellet wet weight *minus* dry weight. Dry weight/pellet is 0.325[14] × the wet pellet weight of an unincubated, untreated sample. The ml cell water/ml pellet water values were obtained by diluting cold cell suspensions with [1-^{14}C]glycine-containing cold diluent, centrifuging immediately, and extracting with cold methanol half saturated with glycine (2 ml/0.2–0.3 g pellet, 3.5 ml/ml medium). 0.1-ml aliquots of extracts were added to 15 ml scintillation mixture (2 g omnifluor, 10 ml Biosolv diluted to 500 ml with toluene) and counted. Quench corrections were unnecessary. The ratio, cpm/ml pellet water:cpm/ml medium is the fraction of pellet water in the extracellular space. This was generally 0.10–0.14, Na_i^+ and Na_0^+ are the measured values for Na^+ in cell water and medium, respectively, in mmoles/l. K_i^+ and K_0^+ have analogous meanings. In Expt 15 the media were prepared with 142 mM Na^+ and 15 mM K^+. After incubation the measured values were $Na_0^+ = 136.6$ mM and $K_0^+ = 18$ mM. In Expt 16, except for the last sample listed, media were prepared with 145.6 mM Na^+ and no K^+; measured values, $Na_0^+ = 145.6$ mM, $K_0^+ = 4.7$ mM. For the last sample listed where no Na^+ and 145.6 mM K^+ were added to the medium, measured values were $Na_0^+ = 0.2$ mM and $K_0^+ = 136.9$ mM.

Expt	*Gramicidin wet weight* (*μg/g*)	Na_i^+	K_i^+	$Na_i^+ + K_i^+$
15	240	117.7	17.8	135.5
	240	128.9	17.9	146.3
	80	124.2	19.9	144.1
	26.7	115.3	18.3	133.6
	8.9	111.3	18.3	129.6
	3.0	112.0	18.2	130.2
16	8.9	139.8	4.2	144.0
	3.0	139.0	6.0	145.0
	3.0	147.2	5.8	153.1
	1.0	140.4	12.0	152.4
	0.32	99.4	56.2	155.6
	0.12	33.8	118.0	151.7
	0.0	21.9	131.4	153.3

gramicidin. In the presence of sucrose, $Na_i^+ + K_i^+$ exceeds $Na_0^+ + K_0^+$ by 18.2±2.1 mM compared to the expected 15.5 mM.

If the Na_i^+ shown in Fig. 1 were not equilibrated with Na_0^+, varying the gramicidin level, and hence the gramicidin-induced component of opposing Na^+ fluxes, would alter the Na_i^+ values. It does not (Table I). Similarly, if K_i^+ were not equilibrated, it should vary with the gramicidin level. Above 3 μg/g cells it does not.

Note that the effect of gramicidin varies as the square of the gramicidin level[18], so a wide range of cation permeation rates was covered.

The equivalence of $Na_i^+ + K_i^+$ and $Na_0^+ + K_0^+ + 1/2$ sucrose (Tables II and III) suggests that the internal cations are osmotically fully active* in gramicidin-treated cells.

TABLE II

EQUILIBRATION OF CELL AND MEDIUM Na^+ AND K^+ BY GRAMICIDIN

Experimental procedure was as described for Table I. Media were prepared with various proportions of Na^+ and K^+ with $Na_0^+ + K_0^+ = 145.6$ mM. The last pair of samples are duplicates where analysis of the media was done only for one of each pair. Except for K_0^+ for the last pair of samples, the concentrations of Na^+ and K^+ added to the media were close to the tabulated (analysed) values. Gramicidin was used at 3 μg/g of cells.

Na_0^+	Na_i^+	K_0^+	K_i^+	$Na_i^+ + K_i^+$	$Na_0^+ + K_0^+$
0.4	2.8	136.2	143.3	146.2	136.6
5.2	18.8	138.3	138.8	147.5	143.5
9.7	17.0	135.4	128.9	145.9	145.0
18.5	21.9	121.8	128.1	150.0	140.3
36.8	31.0	104.6	102.9	133.9	141.4
74.1	65.9	72.1	76.7	142.6	142.6
145.6	139.0	4.7	6.0	145.0	150.3
—	147.2	—	5.8	153.1	—
				Average 145.5	Average 142.8

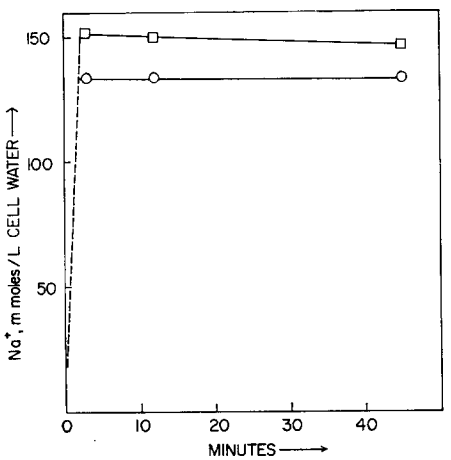

Fig. 1. Effect of gramicidin on cell Na^+ as a function of time. Cells were treated as described in Table I, except that incubation was for the times indicated and in one of the experiments (□) shown here the medium was made hypertonic by addition of 31 mM sucrose.

* This should not be taken to mean that compartmentation is negligible. We have data, although not conclusive indication to the contrary.

TABLE III

EFFECT OF HYPERTONICITY OF THE MEDIUM ON CELL Na^+ AND K^+ EQUILIBRATION PRODUCED BY GRAMICIDIN

Experimental procedure was as described for Table I. Gramicidin was used at 3 μg/g cells, sucrose was 31 mM. 138.3 mM Na^+ and 7.3 mM K^+ were added to the media. Values are given in mmoles/l cell water or medium ± S.E., n is the number of individual samples. The values are averages from 2–4 experiments.

Na_0^+ (mM)	Na_i^+ (mM)	K_0^+ (mM)	K_i^+ (mM)	$Na_i^+ + K_i^+$ (mM)	$Na_0^+ + K_0^+$ (mM)
136.7 ± 1.6 $n=5$	149.5 ± 1.6 $n=6$	6.68 ± 0.16 $n=4$	14.1 ± 3.0 $n=4$	162.0 ± 1.3 $n=4$	143.8 ± 1.6 $n=4$

Effects of gramicidin on glycine accumulation capacity

The effect of gramicidin at 3 μg/g cells on glycine accumulation is illustrated by the experiment of Fig. 2 which is tabulated in Table IV. (The accumulation ratios for its duplicate Expt 22 of ref. 1, are given in the legend of Table IV for comparison.) To avoid possible undesired side effects we used no more gramicidin than necessary

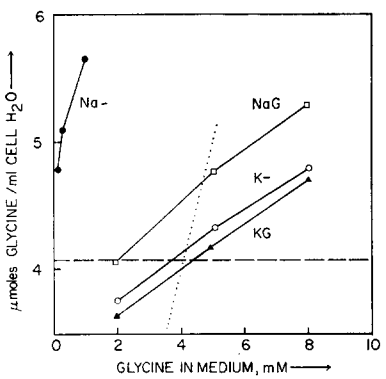

Fig. 2. Effect of gramicidin on glycine accumulation. Ice cold cell suspensions, 2.5% (w/v) in the various incubation media were treated with 3 μg gramicidin/g wet weight of cells as described in Table I. The media had either 138.3 mM Na^+ and 7.3 mM K^+ or 145.6 mM K^+, and concentrations of glycine shown on the abscissa. Media also had 2 mM L-threonine and the sucrose concentrations listed in Table IV. The cation composition of the media is indicated by "Na" or "K" and the presence or absence of gramicidin by "G" or "−". Thus "K−" medium has 145.6 mM K^+, no Na^+, and the cells were not treated with gramicidin. Incubated samples were incubated 45 min at 39 °C, chilled 3 min in an ice bath and immediately centrifuged 10 min at 10000 × g. Pellets and media were extracted with cold trichloroacetic acid, final concentration 5.5% (w/v). Norleucine was added to suitably diluted aliquots of extracts, and the extracts analysed for glycine. Extracellular water was determined, as described in Table I, on duplicate samples representing one each of "KG", "K−", "NaG" and "Na−" which were treated the same as the corresponding samples above except for the external glycine concentrations which are those listed in Table V for Expt 23. The ordinate is cell glycine in mmoles/l cell water after 45 min incubation at 39 °C and the abscissa is the concentration of glycine in the medium. The intersection points of the slant lines with the dashed horizontal line representing initial cell glycine concentration are the points used to calculate accumulation ratios (Materials and Methods). The slant dotted line is the locus of points where cell and medium glycine concentrations are equal.

for cation equilibration. In this experiment, as well as Expt 22 of ref. 1, L-threonine was present in the medium to suppress possible Na^+-dependent, but Na^+ gradient-independent accumulation *via* exchange with endogenous amino acids through the ASC^{24} route. In Na^+ medium without gramicidin, cells can maintain glycine accumulation ratios of 20–40 ("Na−" sample, Table IV and ref. 1). The ratio approaches unity in the presence of gramicidin ("NaG" sample). This ratio is near that with Na^+-free medium in the presence ("KG" sample) or absence ("K−" sample) of gramicidin (Table IV). "NaG" cells still appear to accumulate glycine a little more than "KG" cells. However, in the first of these experiments (Expt 17 of ref. 1) we observed volume changes upon incubation dependent on both the cation composition of the medium and the presence of gramicidin. Such volume changes will affect the apparent accumulation ratio.

TABLE IV

EFFECT OF GRAMICIDIN ON GLYCINE ACCUMULATION

The procedure was as described for Fig. 2. Pellet weights listed are weights after the indicated incubation. "K−", "KG" *etc.* have the same meanings as for Fig. 2. The values, $[Gly_i]/[Gly_o]$, uncorrected, are calculated as described under Materials and Methods. The values, $[Gly_i]/[Gly_o]$, corrected, are values including a correction for swelling. The corrections were made by estimating the 22.5-min values for pellet weights from the swelling curves of Fig. 3 and the initial and final pellet weights in Table IV. From these and values for ml water/pellet and extracellular space, cell water values at 22.5 min of incubation were calculated. Values for ml water/pellet and extracellular space were obtained as described for Table I. Then the initial content of glycine in the cell water of each pellet was divided by the calculated 22.5-min value for cell water and this number used instead of the initial glycine concentration to calculate the "corrected" intersection point (Materials and Methods; Fig. 2), from which the "corrected" $[Gly_i]/[Gly_o]$ values were then obtained. For the duplicate of this experiment, Expt 22 of ref. 1, the uncorrected $[Gly_i]/[Gly_o]$ values were: "K−", 1.14; "KG", 0.73; "Na−", 22.4; "NaG", 1.17. The corrected $[Gly_i]/[Gly_o]$ values, which were not available then, are, in the same order: 0.95, 0.63, 17.5 and 0.98, respectively.

Medium cation	*Medium sucrose (mM)*	*Gramicidin*	*Incubated*	$[Gly_i]/[Gly_o]$ *uncorrected*	$[Gly_i]/[Gly_o]$ *corrected*	*Pellet weight (g)*
K^+	23	0	+	1.09	0.70	0.282
K^+	40	+	+	0.93	0.70	0.300
Na^+	8	0	+	>41.6	>41.5	0.275
Na^+	31	+	+	2.04	1.28	0.298
K^+	0	0	0	—	—	0.302

Cell volume changes and their consequences

Volume changes, measured as pellet weight changes, upon 45 min at 39 °C incubation with gramicidin at 3 μg/g cells were as follows. Cells incubated in K^+ medium with gramicidin ("KG" cells) swelled most (+30.6%, range 28.9–33%, three experiments); "NaG" cells swelled less (+18.6%, range 12.2–29.4%, three experiments); "K−" cells swelled 7.1% (range 4.9–10.6%, three experiments); and "Na−" cells least (+2.8%, range 7.3–(−)5.5%, three experiments). Such volume changes will have effects on $[Gly_i]/[Gly_o]$ unrelated to the energy in cation gradients. The

effects of volume changes on the intersection points (Materials and Methods; Fig. 2) and hence the calculated accumulation ratios are surprisingly large owing to the small slopes of the lines. Note that the ordinate in Fig. 2 is expanded relative to the abscissa.

We could ameliorate but not eliminate swelling by making the incubation media slightly hypertonic with sucrose. Fig. 3 shows wet weights of pellets incubated in such media plotted against incubation time. Each medium had a different sucrose concentration chosen both to minimize the swelling during incubation and to keep the 22.5-min values for pellet weights in the four cases as close together as feasible. These sucrose concentrations were used in Expts 22 (ref. 1) and 23 (Fig. 2, Table IV). The maximum volume change was 31% without sucrose but 12% with sucrose (Fig. 3 and two other experiments, not shown).

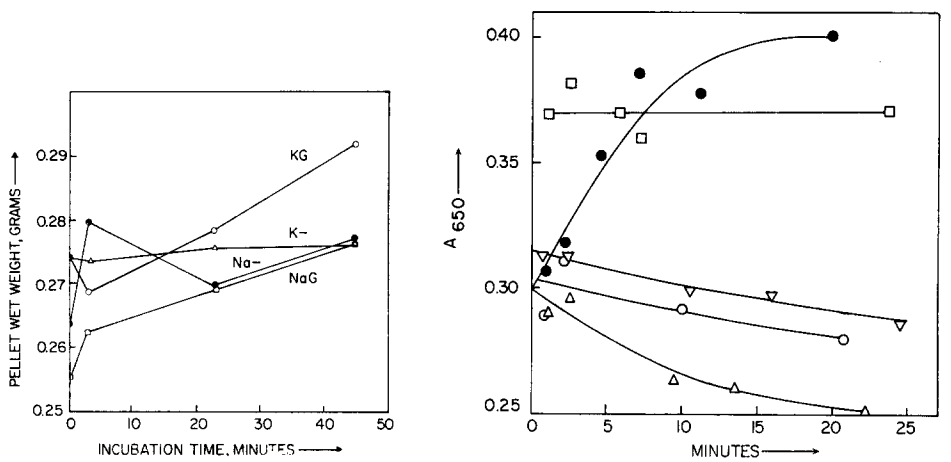

Fig. 3. Effects of gramicidin on cell swelling during incubation with compensatory amounts of sucrose. The procedure was as described for Fig. 2 except that aliquots were taken at various times. The sucrose concentrations were: "K−", 23 mM; "KG", 40 mM; "Na−", 8 mM; "NaG", 31 mM. "K−" *etc.* have the same meaning as for Fig. 2.

Fig. 4. Permeability of cells to Cl^-. 10 ml of a 2.5% (w/v) cell suspension was mixed with 0.05 ml ethyleneglycol and ethanol (9:1, v/w) with or without gramicidin to give 15 or 0 μg/g cells, promptly placed in a 39 °C bath for 3 min and then placed in a 0 °C bath until use. Zero time on the graph is when 0.10 ml of cold 2.5% cell suspension was added to 3.00 ml prewarmed (39 °C) medium. The $A_{650\ nm}$ values were taken at intervals with a Spectronic 20 spectrophotometer. Cuvettes were held in a 39 °C bath between readings. The various media used were "1 × K", "3 × K", and "1 × choline" which had respectively: 10 mM *N*-Tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) (K^+), pH 7.15 buffer *plus* 145.4 mM KCl replacing the potassium phosphate buffer *plus* KCl of the all-K^+ Krebs–Ringer phosphate; 10 mM TES (K^+) *plus* 436.2 mM KCl substituting for the phosphate and KCl; and 10 mM TES (K^+) *plus* 145.4 mM choline chloride as replacements. "G" or "−" are used to indicate the presence or absence of gramicidin. Thus "1 × KG" designates gramicidin-treated cells in an isotonic medium in which KCl is the chief osmotically active component. In "1 × choline" medium, but not "1 × K" medium, K^+ diffuses out because of the K^+ concentration gradient as rapidly as exit of its counter-ion, Cl^-, permits. The rate of cell collapse can be measured by the change in $A_{650\ nm}$ which is predominately due to change in light scattering. This method is modified from that of Scarpa *et al.*[25], being simpler but less accurate. ○, "1 × K−"; □, "3 × K−"; ▽, "1 × choline"; △, "1 × KG"; ●, "1 × choline G".

We attempted to correct the $[Gly_i]/[Gly_0]$ values for the effects of the remaining volume changes as described in Table IV. The corrections appear to bring $[Gly_i]/[Gly_0]$ for "NaG" samples closer to unity and the "NaG" "K−" and "KG" values closer together. The swelling data are too inaccurate and the corrections too crude to allow us to conclude that differential volume changes do account for the differences in glycine accumulation by "K−", "KG" and "NaG" samples. However the volume changes obviously could do so, so these differences do not require the supposition of an alternate energy source.

Chloride permeability

Mammalian red cells are quite permeable to Cl^- (ref. 25). Fig. 4 shows that pigeon red cells are also. Thus there is no Cl^- electrochemical activity gradient from which glycine transport might draw energy. The Cl^- cell/medium ratio prior to gramicidin treatment is near unity (0.83 from data in ref. 26). Thus in untreated cells a membrane potential is not furnishing energy to the glycine carrier by maintaining a Cl^- concentration gradient, and the high Cl^- permeability prevents maintainance of a Cl^- electrochemical gradient. The glycine transport system has an absolute requirement for an anion[27]. If Cl^- is co-transported with glycine, a Cl^- electrochemical activity gradient or concentration gradient, if either were present, could provide energy for glycine transport. We may also eliminate energy contributions from an H^+ gradient as these cells admit H^+ fairly rapidly (Terry and Vidaver, unpublished).

Direct effects of gramicidin on glycine entry

If gramicidin inactivated the Na^+-dependent glycine carrier, accumulation would be abolished regardless of the availability of energy. The data of Table V shows that Na^+-dependent glycine entry, in the presence of gramicidin, is still quite rapid during the last 10 min of a 45-min incubation. Table V also has values calculated for glycine entry on the assumption that entry with or without gramicidin obeys the kinetic equations derived from kinetic studies with hemolysed and restored cells but using an $E_t k_3$ value appropriate for intact cells. These equations embody the Na^+ gradient as the sole energy source.

There are two main points. First, the effect of gramicidin on glycine entry is approximately that calculated assuming gramicidin acts only by raising Na_i^+. Second, the entry rates determined with [1-^{14}C]glycine in the presence or absence of gramicidin are high enough to produce easily measurable changes in cell glycine in 45 min. Thus the inhibition of glycine accumulation by gramicidin is not due to its inactivation of the glycine carrier. Also, since glycine entry is not much affected, inhibition of accumulation must be due to increased glycine exit, the expected consequence of raising Na_i^+.

If gramicidin greatly increased Na^+-independent glycine movement across the membrane, glycine accumulation would be inhibited regardless of the availability of energy. This is not the case. An increased Na^+-independent movement would cause the "KG" curve in Fig. 2 to be rotated, relative to the "K−" curve, counterclockwise about the "KG"–initial cell glycine intersection, toward congruency with the dotted slant line. The latter is the locus of points where $[Gly_i] = [Gly_0]$.

TABLE V

EFFECTS OF INCUBATION WITH GRAMICIDIN ON Na^+-DEPENDENT GLYCINE ENTRY

Cells were treated as described for Fig. 2 with the external glycine concentrations listed in the table except that at approx. 35 min 0.10 ml [1-^{14}C]glycine, 10 μCi/ml was mixed with 10 ml (Expt 22) or 5 ml (Expt 23) of suspension and incubation continued to 45 min. Actual incubation times varied slightly among the samples. The suspensions were then chilled, centrifuged, media and pellets extracted and extracts counted as for cell water/medium water determination described in Table I. "Measured Na^+-dependent glycine entry" is entry into cells incubated in Na^+ medium *minus* entry into cells incubated in K^+ medium but otherwise identically treated. Glycine entry was calculated by dividing ^{14}C taken up in 10 min by the glycine specific activity. No correction was applied for re-exit of isotope during incubation although this is greater for the Na^+-rich gramicidin-treated cells than for the others. "Calculated Na^+-dependent glycine entry" was obtained by using entry rate equation 6c of ref. 17 with the following numerical values for kinetic constants: $K_1K_2K_{G_0}=K_1K_2K_{G_i}=3407$ mM,[3] $K_{G_0}=2.047$ mM, $K_{G_i}=1.381$ mM, $\sigma=0.178$ $\alpha_0=\alpha_1=0.4122$, $K_2K_{G_0}=K_2K_{G_i}=\gamma_0=\gamma_1=\beta_0=\beta_1=0$. E_tk_3 is 11.0 V (entry, $Na_i^+=18$ mM, $Gly_i=5$ mM) in mmoles/l cell water chosen time interval. The value used for V for intact cells, 0.43 mmole/l, 10 min, was that obtained from experiments with Nebraska birds. This V differs somewhat from that obtained[14] with Indiana birds. Values for Na_0^+ and Gly_0 are input values, Na_i^+ values are measured, and Gly_i values were roughly estimated from the interpolated 45-min Gly_i values (*e.g.* Fig. 2), by assuming that at 35 min Gly_i would be $Gly_{i,t=0}+(Gly_{i,t=45}-Gly_{i,t=0})\cdot 35/45$.

Expt	*Gramicidin*	Na_0^+ *(mM)*	Na_i^+ *(mmoles/l cell water)*	Gly_0 *(mM)*	Gly_i *(mmoles/l cell water)*	*Na^+-dependent glycine entry, mmole/l cell water per 10 min*	
						Measured	*Calculated*
22	–	138	18	3.0	5.0	0.29	0.38
	+	138	150	3.0	3.8	0.32	0.48
23	–	140	18	2.0	5.3	0.42	0.38
	+	140	166	2.0	3.8	0.31	0.47
	–	140	18	0.10	3.0	0.20	0.11
	+	140	165	0.10	2.0	0.10	0.12

Effect of gramicidin on cell nucleoside polyphosphates and ATP

If gramicidin caused the destruction of ATP, or some other nucleoside polyphosphate which was the real direct energy source, this effect rather than the abolition of cation gradients could cause inhibition of glycine accumulation. The effects of a 45-min incubation of cells at 39 °C in Na^+ medium in the presence of gramicidin are shown in Tables VIa and VIb. Although there was considerable loss of nucleoside polyphosphate and ATP by the end of the 45-min incubation, this loss was much less than the 94% loss of nucleoside polyphosphate (no apyrase) or the 96% loss (with apyrase introduced into the cells) that was previously found to occur on conversion of intact into hemolysed and restored cells. On such conversion, cells lose only 40% of their glycine active transport capacity[15]. Thus, the loss of cell nucleoside polyphosphates falls far short of accounting for abolition of accumulation capacity. Some loss of cell ATP might be expected since flooding the cells with Na^+ should maximally activate the Na^+–K^+-pump.

TABLE VIa AND VIb

THE EFFECTS OF INCUBATION WITH GRAMICIDIN AND Na^+ ON CELL NUCLEOSIDE POLYPHOSPHATE (VIa) AND ATP (VIb)

Treatment of cells and incubation 45 min at 39 °C were as described in Fig. 2. Nucleoside polyphosphate and enzymatic ATP determinations are described in Materials and Methods. "Recovery factor" is extra nucleoside polyphosphate or ATP recovered from duplicate samples to which ATP had been added just before deproteinization, divided by the amount added. "μmoles *P* per pellet" is the charcoal-adsorbable, acid-labile phosphate. All unincubated pellets were close to 0.28 g. "Fraction P remaining after incubation" is the ratio of the values for the incubated "NaG" sample to that of unincubated "K−" sample. Corrected values are the measured values divided by the recovery factor for the sample. Since the substances recovered are not solely ATP and also since we do not know why the recovery factors vary as much as they do, uncorrected values are also listed in Table VIa. "NaG" samples were, and "K−" samples were not treated with 3 μg gramicidin/g cells.

TABLE VIa

Expt	*Sample*	*ATP recovery factor*	*μmoles P per pellet*		*Fraction of P remaining after incubation*	
			Uncorrected	*Corrected*	*Uncorrected*	*Corrected*
21	"K^+-" unincubated	—	0.60	—		
	"NaG" incubated	—	0.30	—	0.5	—
22	"K^+-" unincubated	0.40	0.74	1.83		
	"NaG" incubated	0.50	0.30	0.60	0.41	0.33
23	"K^+-" unincubated	0.37	1.16	3.18		
	"NaG" incubated	0.45	0.55	1.23	0.47	0.39

TABLE VIb

Expt	*Sample*	*ATP recovery factor*	*μmoles of ATP per pellet (corrected)*	*μmole of nucleoside triphosphate other than ATP per pellet*	*Fraction of ATP remaining after incubation*
24-B	"K−" unincubated	0.67	1.12	0.10	0.32
	"NaG" incubated	0.67	0.35	0.07	
25	"K−" unincubated	0.76	0.97	0.09	0.11
	"NaG" incubated	0.76	0.11	0.07	

Effects of strophanthin k

Experiments with strophanthin were not done in the present study but some previously unpublished data (G. Vidaver) is shown in Table VII. The methods used were those of earlier work[14].

Strophanthin had a minimal effect on glycine accumulation. Even by 60 min (Expt 1) with 10 mg/l strophanthin, the net glycine loss was only 30% greater than the strophanthin-free control loss while the K^+ loss was 400% greater. Note that

under these conditions the uptake component of glycine net loss is sizable. By 23.5 min, [Gly_0] was approx. 0.1 mM from which uptake should be approx. 0.013 mmole/min per l pellet water. The differences between 23.5- and 60-min net losses are consistent with this. Under conditions allowing net glycine accumulation (Expt 2) no effect of strophanthin on glycine uptake was seen. Both strophanthin-poisoned and control cells lost the same amount of K^+. The K^+ concentrations in the media, 0.5 and 1.1 mM before and after incubation, would be too low for the Na^+–K^+-pump of mammalian red cells to act. Here the effects of strophanthin on the Na^+–K^+-pump and cell cations were divorced.

Kimmich[9] has suggested that both Na^+ and organic solutes might reversibly draw energy from a common energy-rich intermediate which is itself energised by ATP. If in our experiments, the high cell Na^+ in gramicidin-treated cells in Na^+-rich media drained the energy from this hypothetical intermediate, gramicidin might inhibit glycine accumulation by such a mechanism. Blocking the Na^+–K^+-pump with strophanthin might either inhibit or stimulate glycine transport depending on the order in which ATP, the hypothetical intermediate, the Na^+ carrier, and the glycine carrier interact. It should do one or the other, however. Strophanthin appears to do neither, so Kimmich's hypothesis appears inapplicable to our system.

TABLE VII

THE EFFECTS OF STROPHANTHIN k ON GLYCINE UPTAKE AND K^+ LOSS

In Expt 1, intact pigeon red cells were incubated with [1-^{14}C]glycine for 20 min at 40 °C in a Krebs–Ringer phosphate medium (Na^+=142 mM, K^+=4 mM), washed and suspended (25%, w/v) in glycine-free Krebs–Ringer phosphate (above) with the strophanthin concentrations listed in the table. Loss of cell glycine at 39 °C (determined both by chemical and ^{14}C measurements) and loss of K^+ was determined from the amounts appearing in the media. The glycine values listed are from ^{14}C data. The chemically determined values agreed well. In Expt 2, intact cells were preincubated 1 h at 40 °C with or without 11 mg/l strophanthin k in a Krebs–Ringer phosphate medium with all Na^+ replaced by K^+, centrifuged, washed and aliquots suspended (10%, w/v) in Krebs–Ringer phosphate with Na^+ as sole monovalent cation and strophanthin and glycine as listed in the table. Cells were exposed to strophanthin either in both incubations or neither. Glycine uptake or loss and K^+ loss upon incubation at 40 °C were measured by chemical determination of glycine in the cells and flame-photometric measurement of K^+ in the media, respectively.

Expt	*Initial glycine in medium (mM)*	*Strophanthin k (mg/l)*	*Glycine uptake (mmoles/l pellet water)*		*K^+ loss (mmoles/l pellet water)*	
			23.5 min	*60 min*	*23.5 min*	*60 min*
1	0.0	0.0	−0.34	−0.49	2.7	1.8
	0.0	0.1	−0.35	−0.54	2.6	5.0
	0.0	1.0	−0.40	−0.66	4.6	7.7
	0.0	10	−0.42	−0.64	5.7	7.4
			20 min		*20 min*	
2	0.0	0.0	−0.32		10.2	
	0.32	0.0	+1.17		9.2	
	0.0	10	−0.32		10.1	
	0.32	10	+1.25		10.3	

TABLE VIII

THE NON-IDEALITY* OF Na^+, K^+ AND WATER

Method	*Tissue*	*Relevant finding*	*Reference*
Ion selective microelectrode	Frog muscle fibers	$\gamma_K \simeq 0.7$, $\gamma_{Na} \simeq 0.1$–0.2	28
	Frog muscle fibers	$a_{Na} \neq [Na^+]$	29
	Barnacle muscle fiber	84% of Na^+, 42% of K^+ "bound"	30
	Isolated cells of bullfrog intestine	$a_{Na}/[Na^+] = 0.45$, $a_K/[K^+] = 1.0$	31
		Solvent water/cell water = 0.84	
Conductivity	Frog muscles fibers	Conductivity 1/3–1/2 that of corresponding saline	32
	Mammalian skeletal muscle, heart muscle, liver, kidney, lung	Conductivity less than external medium	33
	Red blood cells	Conductivity < half expected value	34
	Aplysia neuronal somata	Conductivity < 0.05 of external medium	35
NMR	Rat muscle, rat brain	27 and 13%, respectively, of water more structured than bulk water	36
	Muscle, kidney	65% of Na^+ behaves like Na^+ complexed with ion-exchange resins	37
	Rat skeletal muscle	Minimum of 2 phases of cell water	38
Electron microscope localization of Na^+	Frog skin, kidney tubules	Much more Na^+ in nuclei than cytoplasm	39
	Larus salt gland; chick embryonic myocardium; rat epithelia and Schwann cells; mouse lymphocytes; rat kidney	More Na^+ in nucleus than cytoplasm	4 different groups cited in Table I of ref. 40
Analysis of nuclei dissected from single frozen cells	Frog oocytes	Nuclear/cytoplasmic Na^+ ratio = 2–3	cited in Table I of ref. 40
Radioautography	Frog oocytes	Nuclear/cytoplasmic Na^+ ratio = 3	cited in Table I of ref. 40
Isolation of nuclei in non-aqueous media and analysis	Calf thymus	Nuclear/cytoplasmic Na^+ ratio = 3	cited in Table I of ref. 40
	Calf thymus	Nuclear/cytoplasmic Na^+ ratio = 3	cited in Table I of ref. 40
	Ehrlich ascites cells	Cytoplasmic Na^+ approx. half-average of moles/l cell water	11

* This is not intended to exclude non-uniform distribution of solutes in compartments within which solute behavior is ideal.

The utility of the gramicidin test for other systems

The utility of any test depends on the limitations of alternative tests as well as its own limitations. It is difficult to assess the energy contributed by cation electrochemical activity gradients to solute transport because the relevant cation gradients are not accurately known. Measurements of Na^+ and K^+ activities and, in some cases, water activities have been made in many cell types by several methods in several laboratories. Non-ideality or non-uniform cation distribution has usually been found, especially with Na^+. Studies we have encountered are listed in Table VIII. The ratio, mmoles cation/l cell water is a poor approximation of cation chemical activity. The gramicidin test was designed to circumvent the difficulties in measuring cation chemical activities and the membrane potential.

There are other difficulties besides the inability to measure cation electrochemical activity gradients. One is that prior treatments such as exhaustion of cell ATP by, *e.g.* incubation with dinitrophenol or CN^-, may inactivate the transport system of interest. ATP is required for maintainance of normal red cell deformability[41] and configuration[42]. While not a direct effect of ATP on a transport activity, this ATP requirement for maintainance of normal membrane structure suggests that maintainance of a functional pump might also require energy. Charalampous[43] working with KB cells found that lowering cell K^+ caused a time-dependent loss of the ability to transport α-aminoisobutyric acid. This is an example of a change in the cell internal milieu grossly altering a transport capacity. A direct or indirect dependence of transport capacity on ATP could easily be mistaken for a requirement for ATP to energise transport. Insofar as gramicidin acts quickly and specifically, this problem is mitigated by its use.

Another complication exists because any species co- or counter-transported may contribute to the energy available for a linked transport process. For example, with the pigeon red cell glycine transport system, possible coupled Cl^- and H^+ transport had to be accounted for, and amino acid counter-transport by the ASC route had to be prevented. Complications such as these are not circumvented by the gramicidin test and must be evaluated separately.

ACKNOWLEDGEMENT

This work was supported by grant PHS HE-1325-03 from the U.S. Public Health Service.

REFERENCES

1 Terry, P. M. and Vidaver, G. A. (1972) *Biochem. Biophys. Res. Commun.* 47, 539–543
2 Schultz, S. G. and Curran, P. F. (1970) *Physiol. Rev.* 50, 637–718
3 Jacquez, J. A. and Schafer, J. A. (1969) *Biochim. Biophys. Acta* 193, 368–383
4 Potashner, S. J. and Johnstone, R. M. (1970) *Biochim. Biophys. Acta* 203, 445–456
5 Potashner, S. J. and Johnstone, R. M. (1971) *Biochim. Biophys. Acta* 233, 91–103
6 Lin, K. T. and Johnstone, R. M. (1971) *Biochim. Biophys. Acta* 249, 144–158
7 Johnstone, R. M. (1972) in *Na^+-Linked Transport of Organic Solutes* (Heinz, E., ed.), pp. 51–67, Springer-Verlag, Berlin, Heidelberg and New York
8 Kimmich, G. A. (1970) *Biochemistry* 9, 3669–3677
9 Kimmich, G. A. (1972) in *Na^+-linked Transport of Organic Solutes* (Heinz, E., ed.), pp. 116–129, Springer-Verlag, Berlin, Heidelberg and New York

10 Schafer, J. S. and Heinz, E. (1971) *Biochim. Biophys. Acta* 249, 15–33
11 Pietrzyk, C. and Heinz, E. (1972) in *Na^+-linked Transport of Organic Solutes* (Heinz, E., ed.), pp. 84–90, Springer-Verlag, Berlin, Heidelberg and New York
12 Geck, P., Heinz, E. and Pfeiffer, B. (1972) *Biochim. Biophys. Acta* 288, 486–491
13 Vidaver, G. A. (1971) *Biochim. Biophys. Acta* 233, 231–234
14 Vidaver, G. A. (1964) *Biochemistry* 3, 662–667
15 Vidaver, G. A. (1964) *Biochemistry* 3, 803–808
16 Vidaver, G. A. (1964) *Biochemistry* 3, 795–799
17 Vidaver, G. A. and Shepherd, S. L. (1968) *J. Biol. Chem.* 243, 6140–6150
18 Goodall, M. C. (1970) *Biochim. Biophys. Acta* 219, 471–478
19 Haydon, D. A. and Hladky, S. B. (1972) *Q. Rev. Biophys.* 5, 187–282
20 Mueller, P. and Rudin, D. O. (1967) *Biochem. Biophys. Res. Commun.* 26, 398–404
21 Gomori, G. L. (1942) *J. Lab. Clin. Med.* 27, 955–960
22 (1971) Sigma Tentative Technical Bulletin No. 366-UV, *The Ultraviolet Determination of ATP in Blood or Other Fluids at 340 nm*, Sigma Chemical Co., P.O. Box 14508, St. Louis, Mo. 63178, U.S.A.
23 Darrow, R. A. and Colowick, S. P. (1962) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. V, pp. 226–235, Academic Press, New York and London
24 Eavenson, E. and Christensen, H. N. (1967) *J. Biol. Chem.* 242, 5386–5396
25 Scarpa, A., Cecchetto, A. and Azzone, G. F. (1970) *Biochim. Biophys. Acta* 219, 179–188
26 Vidaver, G. A. (1964) *Biochemistry* 3, 799–803
27 Imler, J. R. and Vidaver, G. A. (1972) *Biochim. Biophys. Acta* 288, 153–165
28 Lev, A. A. (1964) *Nature* 201, 1132–1134
29 Armstrong, W. McD. and Lee, C. O. (1971) *Science* 171, 413–415
30 McLaughlin, S. G. A. and Hinke, J. A. (1966) *Can. J. Physiol. Pharmacol.* 44, 837–848
31 Lee, C. O. and Armstrong, W. McD. (1972) *Science* 175, 1261–1264
32 Katz, B. (1948) *Proc. R. Soc. London, Ser. B*, 135, 506–534
33 Schwan, H. P. and Li, K. (1953) *Proc. Inst. Radio Eng.* 41, 1735–1740
34 Pauly, H. and Schwan, H. P. (1966) *Biophys. J.* 6, 621–639
35 Hovey, M. M., Bak, A. F. and Carpenter, D. O. (1972) *Science* 176, 1329–1331
36 Cope, F. W. (1969) *Biophys. J.* 9, 303–319
37 Cope, F. W. (1970) *Biophys. J.* 10, 843–858
38 Hazelwood, C. F., Nichols, B. L. and Chamberlain, N. F. (1969) *Nature* 222, 747–750
39 Zadunaisky, J. A., Gennaro, Jr, J. F., Bashirelahi, N. and Hilton, M. (1968) *J. Gen. Physiol.* 51, 2905–3025
40 Fry, D. J. (1970) in *Membranes and Ion Transport* (Bittar, E. E., ed.), Vol. 2, pp. 259–276, Wiley-Interscience, London, New York, Sydney and Toronto
41 LaCelle, P. L. (1971) in *The Red Cell Membrane* (Weed, R. I., Jaffe, E. R. and Miescher, P. A., eds), pp. 107–123, Grune and Stratton, Inc., New York
42 Penniston, J. T. and Green, D. E. (1968) *Arch. Biochem. Biophys.* 128, 339–350
43 Charalampous, F. C. (1971) *J. Biol. Chem.* 246, 461–465