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ACTIVE TRANSPORT OF GLYCINE BY MOUSE PANCREAS

EVIDENCE AGAINST THE Na^+ GRADIENT HYPOTHESIS

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SUMMARY

The mouse pancreas when incubated *in vitro* does not maintain a large Na^+ gradient. Despite the near absence of a Na^+ gradient, accumulation of glycine against its concentration gradient is appreciable being 10–12 times that of the medium with 2 mM extracellular glycine. Ouabain, at concentrations less than 0.5 mM, causes a greater perturbation of the steady-state accumulation of glycine than of Na^+ or K^+ distribution. Extracellular Na^+ increases the v_{max} of glycine uptake without a significant effect on the K_m. The extent of glycine uptake can be correlated with the level of cellular ATP and extracellular Na^+ but not with the magnitude of the Na^+ and/or K^+ gradients. The initial rate of glycine uptake is directly proportional to the extracellular Na^+ concentration.

INTRODUCTION

The mammalian pancreas is a remarkable organ. Not only does it combine exocrine and endocrine functions, but it has the greatest protein synthesising capacity amongst adult mammalian tissues with the possible exception of lactating mammary glands[1]. No doubt as an adjunct to this protein synthesising capacity is the ability of the pancreas to transport and accumulate a variety of amino acids[2–6]. Unlike other mammalian tissues which are active in transport, such as kidney and intestinal mucosa, the function has probably evolved primarily to meet the demands for protein synthesis in the pancreas itself rather than a transfer function to supply overall needs in the whole organism.

The ability to establish large differences in amino acid concentration between cell and medium raises the question whether sufficient energy can be derived from the monovalent cation gradients to drive the amino acid transport system. Evidence obtained from studies on intestinal mucosa[7–11] and avian erythrocytes[12,13] has shown that sufficient energy from the Na^+ gradient would be available to bring about the accumulation of sugars and amino acids by a Na^+-dependent mechanism without additional input of metabolic energy, the Na^+ gradient being maintained by the ATP-dependent Na^+ pump. In carcinoma cells an additional contribution of energy from the K^+ gradient must be considered[14–17]. Such a hypothesis has many attractive features coordinating cation transport with that of organic compounds as well as using

the secondary source of energy, the cation gradients, to drive the accumulation of electrically neutral molecules. Thus efficient use is made of all the available energy.

Our recent studies with Ehrlich ascites cells have indicated that the energy from the Na^+ or Na^+ *plus* K^+ gradient(s) does not appear to be used to a large extent to drive accumulation of amino acids against their concentration gradients[18,19]. The present observations with mouse pancreas provide further evidence that the cation gradients are unlikely to drive the accumulation of amino acids against their concentration gradients.

MATERIALS AND METHODS

Male, adult Swiss white mice were used throughout this work. The pancreas, excised immediately after sacrificing the animal, was placed in a chilled petri dish kept on ice. Each pancreas was subdivided into small pieces each weighing approximately 2–3 mg fresh weight. A single incubation flask contained about 20 pieces of tissue (50 mg fresh weight) representative of all the animals used for any particular experiment.

The standard incubation medium contained 145 mM Na^+, 7.8 mM K^+, 1.45 mM Mg^{2+}, 154 mM Cl^-, 10 mM Tris buffer or the buffer indicated in the specific tables and graphs. The gas phase was usually pure oxygen or as specified in the tables. Normally no carbon source was added to sustain respiration as preliminary experiments indicated that addition of glucose or pyruvate did not alter transport activity for 120 min of incubation with fresh tissue.

In all experiments where initial rates of uptake were measured, the tissue was preincubated for 15 min before the radioactive amino acid was added. When uptakes at steady state only were determined, there was no preincubation period.

Accumulation of amino acids by the pancreas was estimated by extracting the soluble amino acids with trichloroacetic acid using the following procedure. After incubation, the flask contents (including tissue) were transferred to tared centrifuge tubes and centrifuged for a few seconds at $800 \times g$. The medium was drained off and the tissue recentrifuged for 2–3 min. The traces of remaining fluid were removed with tissue paper and the tube and contents were weighed. The tissue was homogenized with 3 ml of 5% trichloroacetic acid using a Teflon pestle.

Extraction was allowed to continue for 30 min at room temperature and a fraction of the clear trichloroacetic acid supernatant was counted in a Packard liquid scintillation counter using BRAY's[20] solvent. In a separate series of experiments the relationships between tissue dry weight, fresh tissue wet weight and the change in tissue wet weight after incubation were determined.

Inulin space was used as a measure of extracellular space. After incubation with [^{14}C]inulin for varying periods of time, the [^{14}C]inulin content of the tissue was measured as for ^{14}C-labelled amino acids described above. The extracellular volume was calculated according to KIPNIS AND PARRISH[21].

Early experiments indicated that there is an uptake of water during the course of incubation *in vitro* and this increase in water can be associated with an increased inulin content. To determine the degree of *in vitro* swelling compared to the fresh tissue extra-cellular space, we estimated the inulin space *in vivo* as follows:

The animal was anesthetized with ether, the abdominal cavity exposed, and

the renal blood vessels were tied off with a suture. After closing the abdominal cavity, [^{14}C]inulin was injected from the tail vein. At intervals from 3 to 6 h a heart puncture was made to obtain a sample of blood, then the animal was sacrificed, the pancreas removed and inulin content per mg fresh weight determined in the manner described above.

By 3 h the inulin space per mg pancreas was a constant fraction of the blood inulin concentration. This indicated that distribution of inulin in the pancreas had reached a steady state. Estimation of the inulin content per mg pancreas in this manner showed that the extracellular volume was considerably less *in vivo* than *in vitro*.

Cation estimation

Cations were determined by flame photometry using a Beckman flame photometer. The tissue was extracted overnight at 37° with nitric acid to obtain a clear tissue digest. All values given have been corrected for extracellular space.

ATP was estimated by a coupled enzyme assay[22,23] and phosphocreatine was determined by a modification of the ATP assay in which phosphocreatine kinase was used to synthesize ATP from the ADP added to the assay mixture.

All ^{14}C-labelled compounds used in this work were purchased from New England Nuclear Corp., Waltham, Mass.

RESULTS

The early observations with mouse pancreas indicated that incubation *in vitro* caused considerable increase in the tissue wet weight. This observation is similar to that described by PAPPIUS AND ELLIOTT[24] with rat brain cortex slices. We also observed that the swelling can be accounted for by an increase in the inulin space suggesting that it is extracellular. For operational purposes inulin space was equated with extracellular space and no further experiments were carried out to establish whether the swelling was truly extracellular. The data in Table I show the effects of incubation on alterations in the inulin space. In all subsequent experiments corrections for the appropriate extracellular space have been made.

Examination of the cation composition of the pancreas after *in vitro* incubation, demonstrated that there is a considerable distortion of the monovalent cation distribu-

TABLE I

SWELLING OF MOUSE PANCREAS *in vitro*

All data are the means of two closely agreeing values. Method for determining inulin space *in vivo* given in text. Note agreement between increase in wet weight (line 3) and inulin space (line 6) (values marked with asterisk).

Incubation time (min):	*30*	*60*	*120*
Fresh weight of tissue (mg)	50.7	50.3	50.9
Weight after incubation (mg)	74.6	77.7	78.2
Increase in weight (mg /mg fresh weight)	0.475*	0.540*	0.565*
Inulin space in vitro (μl/mg fresh weight)	0.63	0.70	0.77
Inulin space in vivo (μl/mg fresh weight)	0.188	0.188	0.188
Inulin space increase (μl/mg fresh weight)	0.442*	0.512*	0.582*

TABLE II

GLYCINE UPTAKE AND CATION CONTENT IN MOUSE PANCREAS *in vitro*

Values in parentheses are the number of experimental values. Glycine concentration was 2 mM at 100 counts/min per nmole.

Conditions during 60 min incubation			*Cation content (μequiv/ml cell water) ± S.D.*		*Total glycine uptake (μmoles/ml cell water) ± S.D.*	*Intracellular/ extracellular ratio of glycine*
Medium	*Additions*	*Gas phase*	Na^+	K^+		
Krebs Ringer	Nil	O_2	126 ± 14 (10)	128 ± 13 (10)	22.5 ± 3.7 (10)	11.3
	Nil	N_2	194 ± 3 (3)	69 ± 11 (7)	4.9 ± 0.4 (7)	2.5
	Ca^{2+} §	O_2/CO_2§§§	144; 146 (2)§§	125; 132 (2)§§	21.1; 23.1 (2)§§	11.1
	Nil	O_2/CO_2§§§	126 ± 18 (5)	139 ± 6 (5)	21.0; 22.7 (2)§§	11.0
	Dinitrophenol§	O_2	186 ± 16 (4)	72 ± 9 (4)	9.6 ± 0.9 (10)	4.8
*No K^+	Nil	O_2	207 ± 8 (4)	59 ± 2 (4)	8.4 ± 0.9 (4)	4.2
	Dinitrophenol§	O_2	—	—	4.1 ± 0.1 (4)	2.6
**No Na^+	Nil	O_2	9 ± 1 (4)	60 ± 2 (4)	4.2 ± 0.8 (7)	2.1
	Dinitrophenol§	O_2	—	—	4.2 ± 0.4 (6)	2.1
***Krebs Ringer	40 min pretreatment in N_2	O_2	—	—	22.5; 24.0 (2)§§	11.5
Fresh, unincubated mouse pancreas			13 ± 2 (4)	208 ± 3 (4)	—	—

* K^+ replaced by choline chloride.

** Na^+ replaced by choline chloride.

*** The tissue was preincubated for 40 min under N_2 before incubation at 37° in O_2 to measure uptake.

§ Dinitrophenol, when present, at 0.1 mM; Ca^{2+} at 2 mM.

§§ Duplicate values only.

§§§ $NaHCO_3$ buffer was used. All others Tris buffer.

tion from that observed *in vivo*. It is well known that many tissues suffer a loss of K^+ and an increase in Na^+ as well as an increase in the total cation concentration[25-27]. The pancreas appears to demonstrate this phenomenon to a somewhat greater extent than other tissues examined such as brain cortex slices, kidney cortex slices or liver slices[25-27]. Despite the changes from the *in vivo* situation, the distribution of cations and extent of swelling are very constant and reproducible. The data in Table II show the cation distributions in various media after 60 min of incubation at 37°. The cation contents in fresh tissue are also given.

The data in Table II show that accumulation of glycine is dependent on the metabolic conditions. It is significant to note that although the cellular monovalent cation distributions are similar under N_2, with dinitrophenol in O_2, or in absence of

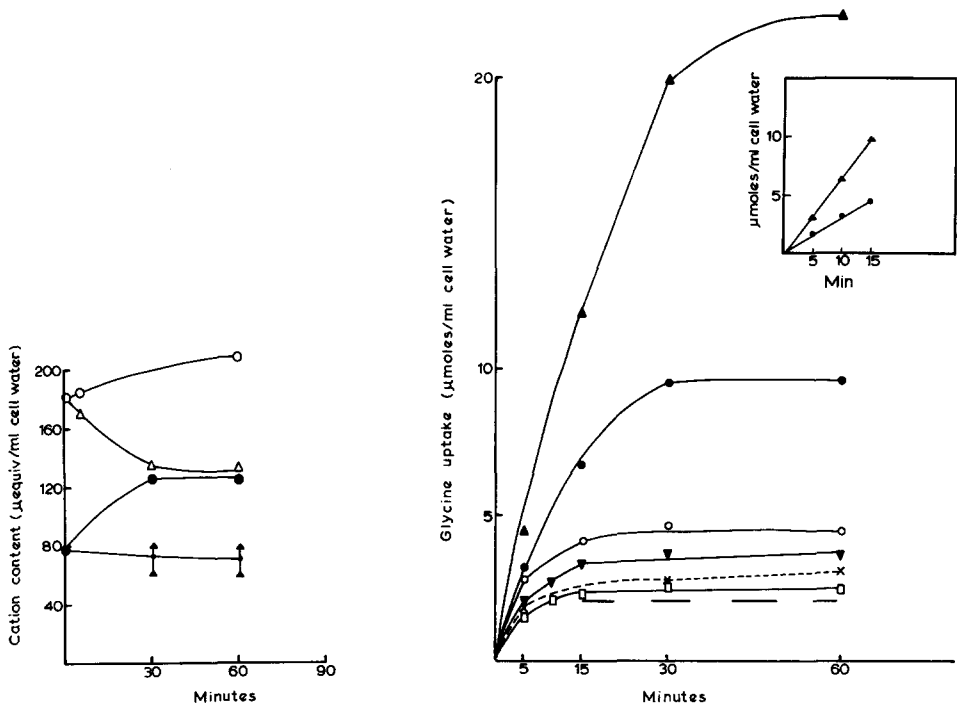

Fig. 1. Change in intracellular cation composition during incubation of mouse pancreas *in vitro*. The tissue was incubated in standard Krebs Ringer medium (see MATERIALS AND METHODS) in a gas phase of pure O_2. The cation content in a sample of tissue was determined before incubation to obtain the initial value. The tissue was incubated with and without 0.1 mM dinitrophenol. Each point represents a minimum of 4 experimental values. The largest variation was obtained with the K^+ determination in presence of dinitrophenol and the spread of values is given. ○—○, Na^+ (dinitrophenol); △—△, Na^+ (control); ●—●, K^+ (control); ▲—▲, K^+; (dinitrophenol). All values corrected for extracellular cations.

Fig. 2. Influence of metabolic conditions on the rate of glycine uptake. The standard incubation medium was used throughout. [1-^{14}C]Glycine (2 mM) was used at a specific activity of 100 counts/min per nmole. ▲—▲, control incubation, O_2 gas phase; ●—●, *plus* 0.1 mM dinitrophenol, O_2 gas phase; ○—○, normal medium, N_2 gas phase; ▼—▼, choline chloride replaced NaCl, O_2 gas phase; □—□, choline chloride replaced NaCl and N_2 gas phase. Thiourea uptake: ×---×, normal medium, O_2 gas phase; ————, theoretical 1:1 distribution Inset: glycine uptake after correction for non-specific component.

extracellular K^+ there are substantial differences in glycine uptake. Under the latter three conditions the uptake of glycine at steady state does not follow the direction predicted from the Na^+ gradient hypothesis[7] being greater in absence of K^+ than under N_2 (compare lines 2 and 6, Table II).

The Na^+ content of the pancreas decreases and K^+ increases for the first 30 min of incubation, reaching 128 and 126 mM, respectively, after 60 min (Fig. 1). In presence or absence of metabolic inhibitors a near steady-state stituation is attained after 30 min incubation. Despite the near absence of a Na^+ gradient at any time ($[Na^+]_o - [Na^+_i] \leqslant 25$ mM) there is extensive accumulation of glycine (Fig. 2). Under the most adverse metabolic conditions (under N_2 and in Na^+-free medium) and within 5 min of incubation, the intracellular concentration of [^{14}C]glycine has reached that of the medium (Fig. 2). Unlike our observations in Ehrlich ascites cells[19] we could not suppress the uptake of [^{14}C]glycine below this 1:1 level by any means and hence it was considered "non-specific". Thiourea equilibrates with the tissue water and its distribution under all conditions is similar to that with glycine under minimal conditions (Fig. 2) (N_2 and in absence of extracellular Na^+). Pretreatment under N_2 does not result in an irreversible loss of transport activity. Indeed after 40 min pretreatment full activity can be restored (Table II).

One of the arguments used to support the Na^+-gradient hypothesis has been the observation that ouabain[10,11] does not affect the initial rate of transport of organic compounds when ouabain and the compound in question are added simultaneously. However, when the tissue is first exposed to ouabain and then the substance to be transported is added, there is a decrease in accumulation. These data have been interpreted as follows: low cellular Na^+ is required to prevent "backflow" of the organic solute when the intracellular concentration becomes equal to or greater than that of the medium. Ouabain acts indirectly on the transport of organic solutes and prior incubation with ouabain is required to effect the dissipation of the cation gradients.

The data obtained with the pancreas, however, are in contrast to those obtained in other systems. The data in Table III show that ouabain causes almost the same percentage inhibition of the initial rate of glycine transport when added simultaneously with glycine or 60 min prior to the addition of glycine. It is possible that the duration of the experimental period (15 min) is sufficient to bring about the maximum effect of ouabain on the cation gradients so that increased time of exposure to ouabain is without additional effect, the action of ouabain, none the less, being mediated

TABLE III

PREINCUBATION WITH OUABAIN ON GLYCINE UPTAKE

The tissue was preincubated in normal Krebs Ringer for the specified time in presence or absence of 0.1 mM ouabain. Then 2 mM [1-^{14}C]glycine at 100 counts/min per nmole was added and incubation continued for 15 min at 37°. Values given are means and range from the mean.

Duration preincubation (min)	*Total glycine uptake (μmoles/ml cell water)*	
	With ouabain	*Without ouabain*
0	9.6 ± 0.2 (2)	14.9 ± 0.1 (2)
15	9.4 ± 0.3 (4)	14.0 ± 0.2 (2)
60	8.1 ± 0.2 (3)	13.3 ± 0.2 (2)

through an effect on the cation gradients. The data in Fig. 3, however, argue against an indirect effect of ouabain on glycine transport. The data show that ouabain, at concentrations of 0.1 mM, decreases glycine uptake at steady state by 50% without a substantial effect on the intracellular Na^+ and K^+ concentrations. Only at higher ouabain concentrations, where glycine uptake at steady state is reduced to near base line values, is the effect of ouabain on the cellular content of Na^+ and K^+ pronounced. It should be noted that the ouabain concentration required to reduce the steady-state cellular K^+ by 50% is nearly 10 times greater than that required to reduce glycine uptake by 50%. With ouabain at 0.4 mM, glycine uptake at steady state is less than that with dinitrophenol, although the cation distributions at steady state are comparable whether dinitrophenol or 0.4 mM ouabain had been present (compare line 5, Table II with Fig. 3). These results suggest that ouabain is acting directly on the amino acid transport system.

The data in Fig. 3 and Tables II and III argue strongly against the likelihood that a Na^+ gradient drives glycine uptake against its concentration gradient. However, this tissue does maintain a K^+ gradient under standard conditions of incubation. We examined the possibility that glycine accumulation was related to the K^+ gradient. In these experiments we found it most convenient to reduce the Na^+ concentration in the incubation medium and to maintain isotonicity with either choline or K^+. The data in Table IV indicate that the K^+ gradient does not contribute significantly to glycine accumulation. If the cation gradient were to influence the accumulation of organic compounds by providing the energy for the process, the effect would be expected to be proportional to the logarithm of the cation gradient and not to the linear gradient. Expressing the K^+ gradient in Expts. I, II and III in logarithmic terms, shows that under none of these three conditions can the uptake of glycine be correlated

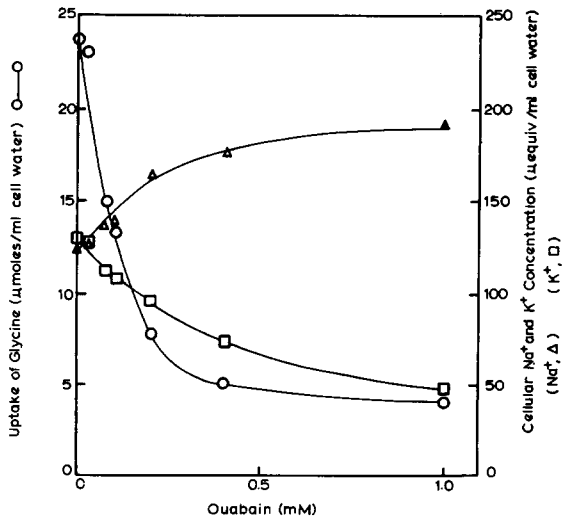

Fig. 3. Action of ouabain on glycine uptake and on Na^+ and K^+ distributions. The pancreas was incubated in standard medium containing 2 mM [^{14}C]glycine with varying ouabain concentrations for 60 min. A 60-min incubation period was used, so that steady-state conditions were examined. ○—○, total glycine uptake; △—△, Na^+ content; □—□, K^+ content. All values corrected for extracellular space and the concentration of solute therein.

TABLE IV

THE K^+ GRADIENT AND GLYCINE ACCUMULATION

Choline chloride was used to maintain isotonicity. Glycine concentration was 2 mM at 100 counts/min per nmole. The values given are means ± S.D. or differences from the mean if only two values. Numbers in parentheses are the numbers of experimental values. Temperature of incubation: Expts. I and III, 37°; Expt. II 25°. Duration of incubation: Expt. I, 30 min; Expt. II, 15 min; Expt. III, 60 min.

Expt.	*Medium cations (μequiv/ml)*		*Cellular cations (μequiv/ml cell water)*		$log[K^+]_i/log[K^+]_o$	*Total glycine uptake (μmoles/ml cell water)*
	Na^+	K^+	Na^+	K^+		
I	48	7.8	58 ± 5 (2)	100 ± 2 (2)	1.10	10.0 ± 0.2 (2)
	48	105.0	52 ± 3 (2)	180 ± 4 (2)	0.23	9.2 ± 0.2 (2)
II	87	7.8	127 ± 2 (3)	93 ± 6 (3)	1.05	3.7 ± 0.3 (2)
	87	65.0	111 ± 4 (3)	165 ± 3 (3)	0.40	3.8 ± 0.3 (2)
III	145	7.8	126 ± 10 (10)	128 ± 13 (10)	1.20 (control)	24.3 ± 1.8 (4)
	145	7.8	158 ± 10 (4)	90 ± 8 (4)	1.05 (ouabain) *	8.0 ± 0.6 (5)

* In Expt. III, ouabain was present at 0.2 mM where indicated.

with the magnitude of the log of the K^+ gradient. The uptake of glycine is largely inhibited by ouabain, despite the presence of a significant K^+ gradient whereas it is unaffected by elevating external K^+ (Expts. I and II) which results in a decrease in the log of the K^+ gradient.

Furthermore, if the cation gradients provide the energy for glycine accumulation, and one Na^+ is transported for every glycine, the potential energy available from the gradients at steady state should always be in excess of the energy required to attain accumulation of the amino acid at steady state[16]. The contribution of an electrical potential difference is not considered in this context since it is known that the potential difference across the pancreatic cell *in vitro* is small (about −10 mV; ref. 28) and is unlikely to have an effect on an electrically neutral substance. That the transmembrane potential contributes little to the energy used for amino acid transport is evidenced by the fact that elevation of extracellular K^+, which decreases the membrane potential, is without effect on glycine accumulation.

Thus

$$2.3\ RT \log \frac{[Na^+]_o}{[Na^+]_i} \cdot \frac{[K^+]_i}{[K^+]_o} \geq 2.3\ RT \log \frac{[glycine]_i}{[glycine]_o}$$

Using the data in Table IV* (Expt. I), it may be seen that when $[Na^+]_i = 48$–50 mM $= [Na^+]_o$ and $[K^+]_o = 105$ mM; $[K^+]_i = 180$ mM; $[glycine]_o = 2$ mM; $[glycine]_i = 9.2$ mM; the product of the left hand side of the equation is log 1.7 whereas the product on the right is log 4.6, a relationship clearly inconsistent with the $Na^+ + K^+$ gradients providing the energy for glycine accumulation, if a 1:1:1 relationship between ions and glycine movement prevails.

We then examined the relationship between cellular ATP and glycine transport. The rate of glycine uptake is constant for the first 15 min of incubation if a "non-

specific uptake" correction is made (inset Fig. 2). Addition of dinitrophenol simultaneously with glycine decreases the initial rate of glycine uptake by about 50% (inset Fig. 2) as well as the uptake at steady state (Fig. 2). During this 15 min period, the cellular ATP level falls from 2.0 to 0.4 mM (Fig. 4.) Pretreatment with dinitrophenol for 15 min, followed by measuring glycine uptake during the subsequent 15 min in the presence of dinitrophenol, results in a 75% inhibition of glycine uptake and the ATP levels fall from 0.4 mM to below detection (less than 0.1 mM). The data show there is a similar pattern in the fall of the ATP level and the ability to accumulate glycine (Fig. 4).

It is unlikely that phosphocreatine provides the energy for glycine accumulation. The levels of phosphocreatine were measured in presence and absence of dinitrophenol. No phosphocreatine was detectable after 15 min exposure to dinitrophenol. The phosphocreatine level in fresh tissue was 1.3 mM. It is apparent that we could not reduce glycine uptake completely even after 2 h incubation in dinitrophenol. The question therefore arises whether this residual uptake is dependent on the existing alkali-metal gradients. Clearly it cannot be due to the Na^+ gradient since the apparent tissue Na^+ concentration is greater than that of the medium (Table II, line 5). Assuming that the intracellular K^+ activity after two hours is equal to the concentration cited in Table II (line 5) sufficient potential energy would be available from the K^+ gradient to bring about a 2-fold accumulation of glycine. The data in Table IV, however, do

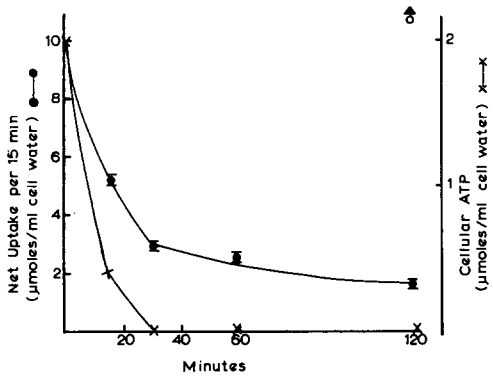

Fig. 4. Decrease in initial rate of glycine uptake with time of exposure to dinitrophenol. ATP concentration in a representative portion of freshly excised pancreas was estimated as well as in portions of the tissue that were exposed to dinitrophenol for 15, 30, 60 and 120 min, respectively. Portions of tissue were also incubated for 120 min without dinitrophenol to determine the maintenance of the cellular ATP level in the control situation (o). The uptake of glycine was measured for a period of 15 min. To measure the effect of pretreatment with dinitrophenol on the uptake of glycine, the following procedure was adopted: The tissue was preincubated with dinitrophenol for 0, 15, 30 and 120 min, after which [1-^{14}C]glycine was added and uptake allowed to continue for a period of 15 min. A control, preincubated without dinitrophenol for 120 min, was also included (▲). Thus, in this figure, glycine uptake after preincubation with dinitrophenol for 15 min actually represents tissue that was exposed to dinitrophenol and glycine simultaneously for 15 min whereas the ATP level is that found at the end of the 15-min period of exposure to dinitrophenol. ●—●, glycine uptake after preincubation with dinitrophenol; ▲, glycine uptake after 120 min preincubation without dinitrophenol; ×—×, ATP level during dinitrophenol treatment; ○, ATP level after 120 min incubation with normal Krebs Ringer medium. All values are means of three or four closely agreeing determinations the spread of which are given for glycine. Values for glycine are net uptakes representing $A_c - A_f$ (cellular concentration less medium concentration).

not support the conclusion that a K^+ gradient is involved, but sufficient data is not available at present to eliminate the possibility completely in ATP-depleted cells.

An equally attractive possibility is that very low levels of cellular ATP support some transport. Additional evidence which suggests that ATP participates in glycine transport comes from experiments with glutamine. The data in Table II show that glycine transport is suppressed to a greater extent under N_2 than with dinitrophenol in oxygen. It has been shown that pancreatic tissue has high concentrations of glutamate[29]. Were glutamate oxidation taking place in presence of dinitrophenol, some ATP production would occur since there is opportunity for "substrate-level" phosphorylation. If this interpretation is correct, addition of small amounts of glutamate or glutamine in the course of pretreatment with dinitrophenol should help to maintain glycine transport under aerobic, but not anaerobic, conditions. Other substrates such as succinate or pyruvate would be ineffective. The data in Table V are consistent with our predictions.

Glutamine is probably more effective than glutamate in supporting glycine uptake because it is known to penetrate mammalian cells more readily. It is unlikely that the enhanced uptake of glycine in presence of glutamine is due to exchange because (1) addition of glycine to cells containing [^{14}C]glutamine does not increase the loss of radioactivity, and (2) the total uptake of glycine in cells first incubated with glutamine exceeds the total uptake of glutamine by a factor greater than two.

If ATP participates directly in amino acid accumulation, the K_m or v_{max} for amino acid uptake may be affected by the cellular ATP level[19, 30]. The results in Fig. 5 show that the maximal initial influx of glycine is decreased by a factor of 6–7 in absence of ATP, whereas the K_m is decreased from about 7 mM with ATP to about 2 mM without ATP. These data are distinctly different from those obtained with Ehrlich ascites cells where the K_m was increased by a drop in the ATP level. The action of extracellular Na^+ is also different from that reported in Ehrlich ascites

TABLE V

ACTION OF GLUTAMINE ON MAINTAINING GLYCINE TRANSPORT ACTIVITY

The pancreas was preincubated in Krebs Ringer medium in O_2 except where indicated otherwise for 120 min with the additions as outlined in the table. Dinitrophenol was used at a concentration of 0.1 mM and glucose at 5 mM. After the preincubation, the gas phase was changed to O_2 where required, [1-^{14}C]glycine, 2 mM, at 88.7 counts/min per nmole was added and the incubation continued for 15 min at 37°. Values in parentheses represent the number of experimental values. The variations are the ranges from the mean value.

Preincubation conditions			*Net glycine transport* ($A_c - A_f$) (*μmoles/ml cell water*) *minus* (*μmoles/ml medium*)
Time (*min*)	*Gas phase*	*Additions*	
120	O_2	Standard medium	9.3 ± 1.5 (4)
		Dinitrophenol	1.1 ± 0.2 (4)
		Dinitrophenol + glucose	1.0 ± 0 (2)
		Dinitrophenol + glutamine (1 mM)	6.7 ± 0.2 (3)
		Dinitrophenol + glutamine (0.5 mM)	4.3 (1)
		Dinitrophenol + glutamate (1 mM)	2.4 ± 2 (2)
		Dinitrophenol + citrate (1 mM)	1.0 ± 0 (2)
		Dinitrophenol + pyruvate (0.5 mM)	1.04 (1)
120	N_2	Glutamine (1 mM)	0.4 ± 0.1 (2)

cells. Extracellular Na^+ appears to alter the maximal initial influx with only a minor (if any) effect on the the K_m value (Fig. 5). Increase in the extracellular Na^+ brings about a proportional increase in the initial rate of glycine uptake (Fig. 6). This effect is reminiscent of that observed by SCHULTZ AND ZALUSKY[34] with carbohydrate transport in rabbit ileum. Thus, both ATP and extracellular Na^+ act primarily on the v_{max} of this system. The data in Fig. 7 show that the initial rate of glycine exodus is unaffected by the cellular ATP level, indicating that the requirement for cellular ATP is associated primarily with the uptake of glycine in this tissue.

DISCUSSION

Under conditions of *in vitro* incubation, the mouse pancreas does not maintain a large Na^+ gradient. After 60 min at 37° the Na^+ gradient is less than 30 mM, being completely absent at the start of the incubation. A significant K^+ gradient is maintained. Despite the nominal Na^+ gradient, there is extensive accumulation of amino

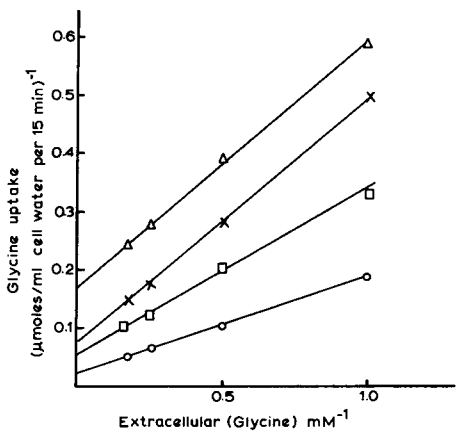

Fig. 5. Action of ATP and Na^+ on the kinetic constants for glycine transport. The pancreas was preincubated for 60 min in normal Krebs Ringer (○—○), 77 mM Na^+ (□—□), 38 mM Na^+ (×—×) and normal Krebs Ringer containing 0.1 mM dinitrophenol (△—△). NaCl was replaced by choline chloride where necessary. After preincubation, sufficient [1-^{14}C]glycine was added to each flask to attain the concentrations cited and incubation continued for 15 min. Each point is the average of 4–8 individual determinations in which the average value is less than ± 5% from the extreme value.

	Kinetic constants	
	K_m (*mM*)	v_{max} (*μmoles/15 min per ml cell water*)
145 mM Na^+	6–7	40*
77 mM Na^+	6–7	18
38 mM Na^+	6–7	13
145 mM Na^+ + dinitrophenol	2.5	6

* or 96.0 μmoles/15 min per g dry wt.

acids. Incubation with dinitrophenol, ouabain or under anaerobic conditions markedly reduces glycine uptake. However, although anaerobiosis, dinitrophenol and ouabain (at 0.10 mM) have comparable effects on the cation distributions, their respective effects on glycine accumulation at steady state show larger quantitative differences, the accumulation of glycine being least under N_2.

Several investigators[7,10,11] have proposed that ouabain acts on the transport of organic compounds by virtue of blocking ion transport and abolishing the cation gradients. Such an interpretation is inconsistent with the present experimental observations since (a) ouabain reduces glycine transport at concentrations which cause little change in cation transport as reflected by the lack of effect on the intracellular cation distributions; (b) no preincubation with ouabain is required to

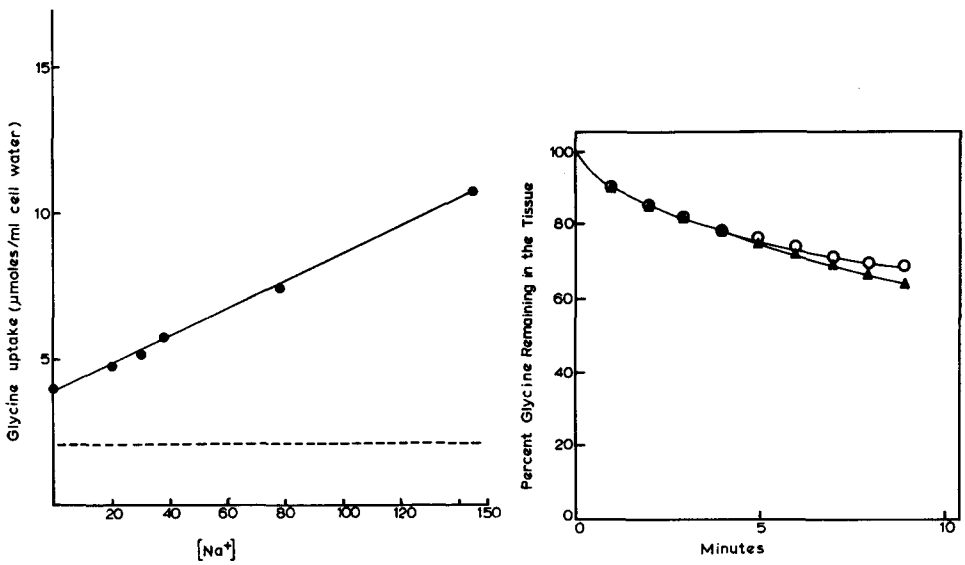

Fig. 6. Action of Na^+ on glycine uptake in mouse pancreas. Pancreas was preincubated for 60 min in media in which Na^+ was replaced by equivalent concentrations of choline chloride to maintain isotonicity and to obtain the Na^+ concentration indicated in the figure. Then [1-^{14}C]glycine to a final concentration of 2 mM was added and the incubation continued for 15 min. The dotted line at the base of the figure gives the medium concentration. The uptake of glycine cannot be suppressed below this level.

Fig. 7. Action of dinitrophenol on initial rate of glycine exodus. The tissue was preincubated with or without 0.1 mM dinitrophenol for 30 min and then [1-^{14}C]glycine was added to give a final concentration of 2 mM (control) and of 8 mM (with dinitrophenol), respectively, in a final volume of 3 ml. The preincubation is essential since the cellular ATP level falls in the first 30 min of the dinitrophenol treatment (see Fig. 4). After the addition of glycine, the incubation was continued for another 60 min. At the end of this period, the cellular glycine content was nearly equal (20 mM) in both cases. To circumvent the problems of unequal extracellular glycine concentrations the following procedure was adopted. At the end of the incubation, the tissue was transferred to tared tubes, spun down, drained and weighed. Then 10 ml of normal Krebs Ringer buffer solution was added to each tube, stirred quickly for 5 sec, spun down again and drained. A portion of this washing medium was counted and it showed more than enough radioactivity to account for the extracellular space. Each tissue was transferred to 9 ml of fresh medium devoid of any amino acid but with and without dinitrophenol as in the preincubation. At various time intervals, 200 μl of the medium were removed and counted. After the final sampling, the radioactivity remaining in the tissue and medium were determined. The total radioactivity associated with the tissue at 0 time of incubation was calculated and used as the 100% control.

obtain maximal inhibition of the initial rate of uptake suggesting that the existing cation gradients do not have to be abolished; (c) nearly 10 times as much ouabain is required to reduce the cellular K^+ by 50% than to reduce glycine accumulation by 50% at the steady state.

That glycine transport is directly dependent on the level of cellular ATP (or some related compound) can be deduced from the following observations.

(a) Reduction of the ATP level by pretreatment with dinitrophenol results in a gradual loss of glycine transport activity. The degree of inhibition of glycine transport by dinitrophenol is related to the time of exposure to dinitrophenol and the inhibition continues to increase after the ions have reached a steady state (30 min).

(b) Glutamine or glutamate, but not pyruvate or glucose, can significantly prevent the effect of pretreatment with dinitrophenol on glycine uptake. This action by glutamate or glutamine is consistent with the ability of the corresponding keto acid, α-ketoglutarate, to support substrate-level phosphorylation. No protection by glutamine is effected under anerobic conditions.

(c) Incubation under N_2 reduces uptake of glycine to a greater extent than incubation under O_2 with dinitrophenol although the effects on cation distributions are comparable. This effect is consistent with observations[29] that freshly isolated pancreas has appreciable levels of glutamate which would provide ATP from substrate level phosphorylation for some minutes of incubation.

Although we have not been able to eliminate the possibility that some other high energy compound, which we have not measured, is the energy source for amino acid accumulation, the present data are also consistent with the interpretation that low levels of cellular ATP can support amino acid transport. If the half-maximal concentration of ATP required for amino acid transport were of the order of 0.5 mM, measurable transport activity would be obtained at concentrations of ATP as low as 0.1 mM and near maximal rates would be obtained at 2–3 mM ATP.

The data reported here are consistent with our earlier observations in the Ehrlich ascites cells[18,19] that cellular ATP, and not the cation gradients, drive amino acid accumulation. The action of extracellular sodium on amino acid transport is in agreement with the many observations in other experimental systems as well as in pancreas[4,5]. A striking observation in the pancreas is the fact that under *in vitro* conditions, ouabain affects the steady state distribution of glycine to a greater extent than the steady state distribution of cations, suggesting that the rate of transport of amino acids may be affected to a greater extent by ouabain than the rate of transport of cations. The greater sensitivity may be due, in part, to the fact that *in vitro* handling has altered the normal cation transport activity so that only a fraction of the normal activity is observed. Nonetheless the fact that glycine transport remains very sensitive to ouabain indicates that (a) glycine transport is not tightly coupled to the normal Na^+ pump activity and (b) ouabain is capable of directly inhibiting transport systems other than the so-called Na^+–K^+ pump.

We propose that ouabain may be an inhibitor of a class of ATPases, which require Na^+ and K^+ for maximal activity. One (or more) of these enzymes may be involved in pumping Na^+ out of the cell whereas others may participate in pumping organic compounds into the cell. The data of ROBINSON[32] have shown that various cardiac glycosides have differential effects on ion pumping and phenylalanine accumulation in intestinal preparations. Furthermore, it has been claimed that ouabain-

sensitive ATPases exist at the brush border of the mucosal surface[33] whereas the pump responsible for Na^+ extrusion is believed to be present at the serosal surface[34,35] of the intestine. Such results are consistent with the present interpretation that a number of cation-requiring, cardiac glycoside-sensitive enzymes exist and may function in the transport of different solutes.

KIMMICK[36] has recently presented data which is in conflict with the Na^+-gradient hypothesis. He argues, however, that a common energy-utilizing mechanism is involved in cation and organic solute transport citing as evidence the fact that K^+ inhibits (competes with) the transport of organic solutes. Although it is clearly premature to state with certainty whether KIMMICK's or our interpretation is correct, three lines of evidence support our interpretation that more than a single ATP-utilizing enzyme is involved in active transport phenomena.

(a) Differential effects on the transport of organic solutes and ions can be obtained with cardiac glycosides.

(b) High K^+ does not inhibit glycine uptake in the pancreas.

(c) In polarized cells, such as intestinal mucosa, there appears to be a spatial separation of the Na^+ extrusion mechanism and the organic solute carrier mechanism[34].

On the basis of KIMMICK's model differential effects of cardiac glycosides are unlikely, and K^+ must compete effectively with the organic solute. In earlier studies by EDDY AND HOGG[37] and our own unpublished studies on Ehrlich ascites cells showed that only at high K^+ (96 mM) and low Na^+ (10–20 mM) did K^+ act as a competitive inhibitor of amino acid uptake. In this regard it should also be noted that for optimal transport of organic solutes low levels of extracellular K^+ are required (see Table II and also refs. 17, 37, 38). High K^+ levels may become inhibitory[37,39,40].

Both Na^+ and ATP appear to enhance glycine transport in mouse pancreas primarily by increasing the maximum uptake. Na^+ in the range from 9 to 145 mM has only a small effect (if any) on the K_m value for the amino acid but raises the v_{max} nearly 10-fold. ATP has a similar effect on v_{max} but in addition increases the K_m value. It is interesting to note that in Ehrlich ascites cells both Na^+ and ATP altered the K_m term whereas in the pancreas both actions are directed chiefly towards the v_{max} term. This relationship may have some bearing on the functional significance of the transport systems in the two types of cells. In Ehrlich cells the amino acid transport system is presumably mainly required to maintain high rates of protein synthesis commensurate with high rates of cell division whereas in the pancreas the high rates of amino acid transport are more likely required to maintain high rates of protein secretion for extracellular functions.

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