AMINO ACID MOVEMENTS IN MOUSE PANCREAS

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Amino Acid Movements in Mouse Pancreas

ABSTRACT

Total movement of amino acids is the sum of simple diffusion, active transport, and exchange diffusion. Various aspects of the latter two processes have been investigated in mouse pancreas in vitro.

(1) The uptake of amino-isobutyric acid and L-lysine over a wide range of concentrations may be explained in terms of one transport site, whereas kinetic analysis of L-tryptophan uptake suggests that at least two sites are involved. In addition to competition among neutral amino acids during transport, there is also competition between neutral and basic amino acids. Uptakes of all the amino acids are inhibited by 0.1 mM 2, 4-dinitrophenol to about the same extent. The uptake of several neutral amino acids is scarcely sensitive to pH, while the uptake of L-lysine (basic) is significantly decreased at low pH.

(2) Sodium and potassium ions affect the apparent affinity constant without altering the maximum concentration gradient. Kinetic analysis indicates a 1:1 relationship between sodium ions and molecules of 1-aminocyclopentanecarboxylic acid transported into mouse pancreas. In low sodium or potassium media the uptakes of amino-isobutyric acid, 1-amino-cyclopentanecarboxylic acid, glycine and L-methionine are decreased to approximately the same extent. Uptake in the absence of external sodium is still concentrative and is inhibited by 2,4-dinitrophenol and other amino acids, but not by ouabain.

The increased fluxes of amino acid in response to different amino acids on the opposite side of the membrane are due to exchange diffusion. They are characterized by: (a) a definite substrate specificity, (b) no dependence on the concentration of external sodium (c) affinity constants which are similar to those characterizing the transport process, and (d) interaction between amino acids which may result in a greater exchange in the presence of two amino acids than in the presence of either alone or in an inhibition during transport but not during exchange diffusion.

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by

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LIST OF ABBREVIATIONS

ATP	adenosinetriphosphate
DNP	2, 4 dinitrophenol
TCA	trichloroacetic acid
ACPC	1 - aminocyclopentanecarboxylic acid
AIB	α - aminoisobutyric acid
DOPA	dihydroxyphenylalanine
MeACPC	1-amino-2-methylcyclopentanecarboxylic acid
GABA	γ - aminobutyric acid
Na^+	sodium ions
K^+	potassium ions
Ca^{++}	calcium ions

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CHAPTER 1

INTRODUCTION

1.1 Introduction to Transport and Transport Theories

The concept of the role played by the cell membrane in the movement of biological materials has changed very drastically. It was originally thought to be merely a static, inert barrier between the extracellular and intracellular fluid compartments, but it is now quite evident that the membrane possesses certain components, as yet unidentified, which participate in the translocation of substances from one fluid compartment to the other. They have been arbitrarily designated as "carriers" and it is via attachment to such "carriers" that electrolytes and non-electrolytes are thought to enter cells in addition to the processes involved in simple diffusion.

The presence of a functioning carrier can result in either of two processes. The first, termed "facilitated diffusion" (1) involves simply a diffusion phenomenon which is facilitated by a membrane carrier. It requires no energy to maintain and results in an intracellular substrate concentration not exceeding that of the external environment. In contrast, the second process, that of active transport, involves not only carrier-mediated movement, but also a definite energy requirement. As a consequence there is a greater intracellular than extracellular substrate concentration. Csaky (2) has defined active transport as "an energy requiring process whereby a substance permeates across plasma membrane

or membranes from a lower into a higher concentration, yet the substance is neither bound on either side of the membrane nor produced during the transport process nor passively carried by the movement of the solvent."

There are two lines of evidence in support of the membrane containing specific pumping mechanisms. For example, reconstituted erythrocyte ghosts can be prepared which transport Na^+ and K^+ uphill if ATP is present in the ghost (3). The Na^+ and K^+-dependent ATPase has been shown to be associated with the membrane (4, 5). Secondly, protoplasts from E.Coli, in which a β-galactoside "permease" has been induced, transport galactosides against high concentration gradients (6).

The transport model of Heinz and Walsh (7) proposes that the activated mobile carrier first forms a complex with the extracellular amino acid and then moves towards the interior of the cell where some of the amino acid is released, while the remainder is exchanged with available intracellular substrate. The "empty" carrier is then inactivated in order to prevent its outward movement. This is essential if accumulation is to occur. Jacquez and Sherman (8) have suggested that energy is linked to the carrier system in order to bring about an increased rate of outward movement of free carrier or an increased dissociation of amino acid-carrier complex at the inner portion of the membrane. Heinz (9), taking into account the transport model of Mitchell (10), has proposed a mode of entry which does not require the carrier to be mobile. In this scheme an intracellular acceptor is activated prior to combination with an enzyme in the membrane to form an enzyme-acceptor-complex. This complex then

reacts with the external amino acid to form an amino acid-acceptor-complex which is released into the interior of the cell where it dissociates to produce free amino acid and acceptor molecules. Patlak's "gate" model for biological transport (11) proposes that a structural change may occur when the solute binds to the "gate" in the membrane and as a consequence the solute is released more readily into the interior of the cell.

With regard to the nature of the membrane factor, some proteins have been separated from bacteria which are apparently related to the transport of sulphate in Salmonella typhimurium (12), β-galactoside in E.Coli (13, 14) and the amino acids leucine, isoleucine and valine in E.Coli (15). Proteins could be dislodged from positions on the membrane by means of osmotic shock (12, 16).

Christensen (17) has suggested that there should be ".... a shift of attention from the carrier concept toward the concept of a reactive site on a protein molecule in the membrane, unencumbered by an insistence on its having enzymatic capabilities." He further argued that ".... attention should focus on the changes in conformation of which such proteins are capable .... rather than on the possibility that a diffusible enzyme-substrate complex is formed." Since the protein, hemoglobin shows on binding oxygen, a shift of $7A^{o}$ in the position of other binding sites on the molecule with relation to each other (18), Christensen (17) believes that perhaps "this action may provide as good a model of the molecular behavior that may be required for directed transport .... ".

1.2 Historical View of *in vivo* Transport

Most of the early studies involved an *in vivo* approach to the transport problem. In 1913 Van Slyke and Meyer (19) concluded, that, since the concentration of amino nitrogen was five to ten times higher in the tissues than in the blood, osmosis could not be the only process by which amino acids were taken up by the tissues. Friedberg and Greenberg (20) found that intravenously administered amino acids which were rapidly removed from the plasma, were greatly concentrated in liver and kidney, and to a lesser extent in skeletal muscle. The effects of fed amino acids upon the distribution of other amino acids between the interior and exterior fluid compartments led Christensen *et al* (21) to conclude that "amino acids compete with each other .... for the means by which cells concentrate the amino acids presented to them by the extracellular fluid."

The concept of a "selective permeability" of the small intestine was developed through the early experiments of Hober (22), Hedon (23) and Nagano (24) which showed that sugars were absorbed at very different rates. Cori (25) suggested that amino acid transfer could not be due to simple diffusion since the rate of absorption from a mixture of two amino acids was not additive. Examining the rates of absorption of amino acids from the gastrointestinal tracts of rats, Kamin and Handler (26) found that the presence of an excess of one amino acid almost invariably inhibited the intestinal absorption of another amino acid. Further evidence against simple diffusion came from the work of Hober and Hober (27) who, by studying the kinetics of intestinal absorption in rats, showed the

absolute rate to be independent of the concentration. They concluded that a specific transport mechanism must be involved. In addition to interaction between similar substrates, it was also suggested that amino acids and sugars might be mutually inhibitory during intestinal absorption, since there was mutual inhibition in the absorption of mixtures of glucose and glycine given to rats (25).

Brain could also accumulate amino acids in vivo to intracellular levels exceeding those of the plasma (28-30). Kamin and Handler (31) noted that perfusion of anesthetized dogs with the dibasic amino acid lysine caused an increase in brain lysine. Injection of leucine-C^{14} into mice, resulted in such a rapid increase in the specific activity of brain leucine, that this value exceeded the plasma activity after only five minutes (32). An intraperitoneal injection of the L-isomer of tyrosine also resulted in an increased concentration of this amino acid in the brain (33). Since the uptake could be inhibited by a variety of amino acids, the authors concluded that the in vivo uptake of amino acids was due to specific mechanisms rather than simple diffusion.

The administration of proline intracisternally rather than intravenously increased the proline concentration in the brain (34), while intravenous injection of proline ethyl ester also resulted in an elevated level of proline (35). This was possible due to hydrolysis of the proline ester to free proline in the brain. In addition, several comparison studies with glutamic acid (36), lysine (37) and leucine (38) indicated that the in vivo uptake of these L-amino acids by the brain was greater

in the newborn than in the adult. The *in vitro* aspect of transport in brain began with the demonstration by Stern *et al* (39) that brain cortex slices from guinea pigs could accumulate L-glutamate against a concentration gradient *in vitro.* Other *in vitro* studies will be discussed in a later section of this literature review.

Reabsorption by the kidney tubules *in vivo* has also been the subject of numerous investigations. Kriss (40), studying the partition of urinary nitrogen following the oral administration of various amino acids, suggested that all may not be reabsorbed with equal efficiency. Subsequent work by Doty (41) indicated that certain amino acids, although structurally similar, may be reabsorbed at widely different rates. Competition for reabsorption by the renal tubules was observed following the intravenous administration of such amino acid pairs as arginine and lysine, arginine and histidine, and leucine and isoleucine (42). Competition for reabsorption could not, however, be demonstrated with the amino acid pairs arginine and glycine, leucine and glycine, isoleucine and glycine, and arginine and leucine (42).

Pitts (43) found that glycine was reabsorbed by a transport mechanism which exhibited, as did the glucose reabsorptive system (44), a limited transfer capacity. Creatine was reabsorbed by the same mechanism as glycine and so these two amino acids, when present together, competed for the common reabsorptive system (43). Comparison of the renal reabsorptive processes for several amino acids in the dog, led Pitts (45) to conclude that " the amino acids glycine, alanine, glutamic and arginine

are in all probability reabsorbed by a common mechanism and that the rather considerable variations in their rates of reabsorption result from differences in their rates of combination with some cellular component common to the reabsorptive system for all." It was only later that separate, distinct transport systems for neutral, basic and acidic amino acids were shown to exist in the kidney. Much work has also been concerned with hereditary disorders of renal tubular reabsorption and these will be discussed in the section dealing specifically with amino acid transport in kidney.

Prior to the *in vitro* work of Bégin and Scholefield (46, 47, 48) a great many *in vivo* studies had been carried out with pancreas. Wheeler *et al* (49) showed that when S^{35}-methionine was given intravenously there was accumulation in the pancreas as well as in the small intestine and liver. This was confirmed by Hanson and Ullberg (50) who noted that S^{35}-methionine was also highly concentrated in other organs such as kidney, spleen, bone marrow and salivary glands. Several other amino acids such as S^{35}-DL-cystine, C^{14}-DL-phenylalanine and C^{14}-glycine were similarly accumulated in the pancreas of mice (50). By means of autoradiographic studies, Leblond *et al* (51) were able to demonstrate that the exocrine portion of the pancreas was mainly responsible for the high uptake of labelled amino acids. This was later confirmed *in vitro* by Bégin and Scholefield (46) who found that the β-cells of the pancreas could be destroyed without any significant change in the amino acid uptake.

By means of whole body autoradiography (52, 53), it was shown

that ^{14}C-ACPC, an amino acid which undergoes no metabolic breakdown (54), was concentrated selectively by the pancreas and bone marrow of normal mice (55). The concentration of this isotope by mouse pancreas was confirmed by Sherman et al (56) who also noted that ACPC was not accumulated by the pancreas of either dogs or rabbits. Rats on ACPC diet showed higher levels of ornithine, lysine, glutamic acid, glycine and phenylalanine in the serum (57). It was suggested that ACPC, which readily penetrated the cells against a concentration gradient, could displace other amino acids previously contained in the cells. Furthermore, other amino acids such as dihydroxyphenylalanine-2-C^{14} (58), C^{14}-DL-para-Fluorophenylalanine (59), ethionine (60), and DL-tryptophan-C^{14} (61) were similarly accumulated by the pancreas as well as by several other organs.

The ability of the pancreas to accumulate injected amino acids has become an important diagnostic tool in the field of medicine. Se^{75}-selenomethionine has sufficient pancreas specificity to be used for visualization of the organ by isotope scanning in order to detect any existing lesions (62). The concentration of radioactivity in the pancreas was about 8 or 9 times higher than the liver when Se^{75}-selenomethionine was given just before or just after feeding, while the concentration in the other viscera and in blood were lower than in liver (61). Starvation appeared to increase the concentration of the isotope in the liver, causing a reduction in the pancreas to liver ratio.

1.3 General Features of Amino Acid Transport

(A) The Effects of Concentration

In Ehrlich ascites cells, elevation of the extracellular amino acid concentration resulted in an increased intracellular concentration as well as a decrease in the distribution ratio (intracellular/extracellular). With increasing glycine levels "the relation is curvilinear, neither the distribution ratio nor the gradient being constant over the range illustrated" (63).

The uptake of glycine obeyed the Michaelis-Menten equation and could be represented by (64):

$$V^{o}_{max} \quad \frac{A_f}{K_m + A_f} = K_{diff} (A_c - A_f)$$

where

V_{max} = maximal rate of transport,

A_c & A_f = intracellular and extracellular glycine concentrations respectively,

K_m = the Michaelis constant,

K_{diff} = the diffusion constant across the cell boundary.

At the steady state distribution the equation becomes:

$$\frac{A_c}{A_f} = \frac{A}{K_m + A_f} + 1$$

in which A is a constant, representing V^{o}_{max}/K_{diff}.

This equation could be arranged to the simplified form:

$$\frac{A}{A_c - A_f} = 1 + \frac{K_m}{A_f}$$

The heat of activation of the initial rate of uptake of glycine was 8600 calories (64), in contrast to that of free diffusion which was 4500 calories (65). This supported the argument that glycine uptake was mediated by binding to some cellular component and not due to a diffusion type of entry (64).

Application of the laws of enzyme kinetics to amino acid transport led Scholefield (66) to look at the interaction between amino acids in Ehrlich ascites cells according to the equation:

$$\frac{A}{A_c - A_f} = 1 + \frac{K_m}{A_f} \left(1 + \frac{I}{K_I} \right)$$

Using this formula good agreement between actual and theoretical values was observed, indicating that the competition between amino acids was of a competitive nature and that principles of enzyme kinetics could be applied to amino acid transport.

(B) The Effects of Temperature

Transport processes generally show the temperature dependency characteristic of enzyme-catalyzed reactions. For example, Oxender and Christensen (67) have found a Q_{10} for glycine of 3.5, for alanine 3.2, AIB 3.5, leucine 2.5 and valine 2.7 in Ehrlich ascites cells, while the Q_{10} for L-tyrosine uptake in isolated rat diaphragm was 1.4 between 27 and

37°C (68). The uptake of L-tyrosine in sarcoma 37 ascites cells had a Q_{10} of 1.9 when measured between 17 and 37°C (69). Tenenhouse and Quastel (70) pointed out that the Q_{10} for glycine uptake was 1.5 between 10 and 20°C, and 2.41 between 25 and 37°C, thereby emphasizing that the Q_{10} is dependent upon the temperature range used for measurement.

The optimal temperature for the active movement of amino acids differs from tissue to tissue. For instance, the optimum temperature of uptake of tyrosine by diaphragm is 38°C (68), whereas the optimum for L-S-ethylcysteine in Ehrlich ascites cells is 25°C (70). In addition, incubation of 6C3HED ascites cells, in an ACPC containing medium at 26°C and 37.5°C resulted in identical intracellular amino acid concentrations at both temperatures (71). The decreased uptake of amino acids by elevation of the temperature to 40°C or higher has been suggested to be due to some non-specific change in the cell membrane (68).

A decrease in temperature below 15°C resulted in a very markedly reduced active transport process in Ehrlich ascites cells (70), while in brain slices some amino acids were concentrated to levels slightly above that of the external medium even at 0°C (72). This suggested a distinct carrier-mediated accumulation even at this low temperature.

From kinetic studies on the influx of radioactive glycine into Ehrlich cells, Heinz (64) calculated the apparent heat of activation from the temperature coefficient between 24°C and 32.5°C to be about 8600 calories. This is much greater than the 4500 calorie heat of activation of free diffusion (65). Heinz (64) also demonstrated, using glycine,

that the K_m value increased as the temperature was lowered from 37°C to 28°C, thereby resulting in the carrier being less saturated at this temperature.

In addition to transport, exchange diffusion is also affected by temperature changes. For instance, Ehrlich ascites cells required a much longer period of time at 20°C than at 37°C for L-methionine and L-ethionine to exchange completely (73). In addition, there was a decreased efflux of preloaded amino acid as the temperature was lowered.

(C) The Time Course of Uptake

Heinz in 1954 (64) demonstrated that the relative uptake of glycine into the Ehrlich cell follows the equation:

$$\mu = U_{\infty} (1-e^{-kt})$$

where

μ = uptake

U_{∞} = the maximal uptake

k = a constant

t = time in minutes

There was a very rapid initial uptake of glycine into the cell, although the time required to reach half the steady-state value has varied from 3 minutes (64) to 20 minutes (67, 74).

In brain slices the initial uptake of L-alanine was greater than the D-form, although at the end of 60 minutes the intracellular concentration of D-alanine was approximately double that of L-alanine (75).

With DL-∝ AIB and ACPC uptake the initial rate increased rapidly resulting in about 70% of the final level in 15 minutes with a steady state in 60 to 90 minutes (76), while the active accumulation of D-glutamate and GABA occurred linearly over the period of an hour incubation. This was accompanied by a gradual decrease in the intracellular concentration of these latter amino acids over a period of 4 hours (77).

The extent of the initial rate is not always reflected in the steady-state situation. For example, although in kidney slices glycine had both the highest initial and steady-state rate when compared to ∝ AIB, histidine and L-phenylalanine (78), Christensen et al (79) have shown that although ∝ AIB had 1/3 the initial rate of uptake of L-methionine in the Ehrlich cell, its distribution ratio after 30 minutes was twice that of L-methionine. In addition, Finch and Hird (80) with intestinal segments have demonstrated that amino acids with the same initial rate can have different steady-state levels.

The time required to reach the steady-state level of amino acid has varied from tissue to tissue, with 30 minutes required for glycine in Ehrlich cells (81), 90 minutes in kidney slices (78), and 60 minutes in mouse pancreas (46). In the case of ∝ AIB uptake in brain (76) equilibrium was reached between 60 and 90 minutes, whereas in fetal rat calvaria the steady-state level was attained only after 4 hours (82).

(D) The Effects of pH

By plotting glycine influx into Ehrlich cells against pH,

Heinz (83) obtained a titration curve with an inflexion point at about pH 6.9. Since the inflexion point was not shifted by increasing glycine concentration, Heinz concluded that "there was no competition between substrate and H^+ ion for the same point of attachment". The uptakes of leucine and phenylalanine into the Ehrlich cell were relatively insensitive to pH, whereas α AIB and alanine accumulation were affected much more by pH changes in the external environment (67). In erythrocytes and reticulocytes, the uptakes of valine and alanine were affected by pH (84), but the contrast was not as striking as that observed with the Ehrlich cell (67). Guroff and Udenfriend (68), examining tyrosine uptake in isolated rat diaphragm, showed that although a pH of between 7.0 and 8.0 generally gave slightly lower values than those between 6.0 and 7.0, the uptake was relatively insensitive to pH change.

Although a large number of amino acids reach higher intracellular levels at slightly basic pH values (85, 67), the uptakes of α,α-diethylglycine and α,α-dipropylglycine by the Ehrlich cell were greater at pH 5.0 than at pH 7.4 (86). Glutamate transport in Ehrlich cells was also stimulated at acidic pH values (87). This was explained as being due to protonization at or near the transport site, which in the protonized state has a higher affinity for glutamate. The authors showed that the effect of pH was on the active and not the passive portion of uptake, since the glutamate stimulation was not obtained when there was metabolic inhibition by DNP or when the incubation was carried out at $1^{o}C$. This lack of effect of acidic pH on passive entry has been confirmed by Kromphardt (88).

(E) Structural and Configurational Requirements for Transport

In the Ehrlich cell the structural features which participate in the bonding of neutral amino acids to the transport mediating system are the amino group, the carboxyl group and the nature of the side chain (67). Neutral amino acids were accumulated in these cells whether the amino group was α or β relative to the carboxyl group (89, 90), while loss of the amino group resulted in compounds which were not actively concentrated. Amino acids such as proline and hydroxyproline were still transported, indicating that the amino group may be removed from a primary position by ring formation (91). Those possessing a methylated amino group still could be transported, although this action resulted in particular amino acids losing reactivity with certain routes by which they entered the cell (92). Replacement of the carboxyl group by a sulfonic acid grouping also resulted in accumulation (93), while complete loss of the carboxyl group resulted in amines which were transported in the Ehrlich cell (94), as well as in the mast cells of the rat (95, 96). With respect to the side chain, increasing or branching caused a decrease in the ability of the particular amino acid to be concentrated (91).

Examining the amino group requirement in intestine, Spencer et al (97) found that glycine but not acetic acid was transported, thereby indicating that the amino group was essential. One hydrogen of the amino group could be replaced, although if the replacement group was too bulky, the resulting compound was not transported. Since α L-alanine, but not β -alanine was accumulated, they concluded that it was essential to have

an amino group γ to the carboxyl. In order not to have a pronounced effect on the transport rate in hamster intestine, the carboxyl group must be free, the amino group must not exist in a tertiary or quaternary state, and both hydrogens must not be replaced by methyl groups (98). It was advantageous that the side-chain be of a non-polar nature, although there was relatively little restriction on its structure in order for transport to occur. In the case of cyclic amino acids of the form $(CH_2)_n$ NHCHCOOH, there was transport against a concentration gradient when n was 2, 3 or 4, but not when n = 1 (99). With non-cyclic amino acids of the form $Y\text{-}\overset{X}{N}CH_2COOH$, (when X=H) transport occurred with Y = CH_3, but not with any larger groupings (99).

The D- and L-amino acid configurations behave quite differently in a number of tissues. For example, in brain (75) the concentration ratio obtained with L-alanine was initially higher than the D-form, although after a longer incubation period was lower than the D-form. Comparison of the transport of the L- and D-isomers of lysine and leucine in newborn and adult brain slices showed that the L-isomer uptake was higher, except in newborn slices where the D-isomer of leucine was accumulated to a greater extent (76). In Ehrlich ascites cells (100) the L-isomer of tert-leucine was very rapidly concentrated, whereas with the D-isomer it was difficult to demonstrate a concentration gradient after 30 minutes. In the case of S37 ascites cells, the D-isomer of tyrosine appeared to be transported to a greater extent than the L-form (69).

Using isolated rat intestine, Agar et al (101) noted that

the L- but not the D-forms of histidine and phenylalanine were accumulated against a concentration gradient. Wiseman (102) found that the L-isomers of alanine, methionine and isoleucine could produce concentration gradients, while the transport of the D-isomers could not be demonstrated. The L-isomers of histidine and alanine were absorbed at a more rapid rate than the respective D-isomers from the isolated guinea pig intestine (103). Furthermore, Agar et al (104) found that the uptake of L-histidine was inhibited by L- but not D-amino acids at a similar concentration. Finch and Hird (105) concluded that "... studies with D-amino acids suggest that they combine with the site for uptake of L-amino acids, but have a lower affinity for it".

(F) The Role of Energy

The active accumulation of amino acids has been shown to be dependent upon a source of energy. In the Ehrlich cell changing from aerobic to anaerobic conditions, resulted in a decreased glycine uptake, while incubation with compounds such as DNP, cyanide, iodoacetate also caused a decreased intracellular level of amino acid (63). A 0.1 mM concentration of DNP inhibited the uptake of glycine by 50% and S-ethyl-cysteine by 40% with no significant effect on transport under anaerobic conditions, while iodoacetate inhibited the anaerobic transport of these two amino acids with no significant effect on the aerobic transport (70). The authors concluded that the "concentrative uptake of glycine and S-ethyl-cysteine requires energy which can be supplied by respiration or under anaerobic conditions, by glycolysis".

In brain slices 1 mM DNP markedly suppressed glutamate transport (39) and tyrosine transport (106), although 0.05 mM DNP had little effect on glutamate uptake (107). Subsequent work with brain indicated that the extent of glycine accumulation was directly proportional to the level of ATP and metabolic inhibitors which caused a decreased ATP level also resulted in a decreased glycine uptake to approximately the same extent (108).

The response of amino acids to inhibitors of energy metabolism vary considerably as shown by little or no effect of 0.05 mM DNP on glutamate accumulation by rat brain contex slices (107), while glycine transport was inhibited to the extent of 70% by only 0.02 mM DNP (108).

In brain slices the greatest ATP content and uptake were observed with glucose and mannose in the medium, although slightly depressed uptakes in the presence of other compounds were obtained (109). This is in contrast to the findings of Bégin and Scholefield (46) in mouse pancreas, where the addition of many different substrates had little or no effect on either the respiratory activity or the ability to concentrate amino acids.

The addition of DNP to Ehrlich cells previously labelled with P^{32}-phosphate caused a rapid loss of radioactivity from ATP within the first 5 minutes (110). Other work (81) showed that when 0.1 mM DNP was added to ascites cells after the cells had attained about 50% of the steady state with respect to glycine, radioactive glycine entered the cells at the same rate for the first 5-10 minutes as the cells in-

cubated in the absence of DNP. More recently Conway (111) has proposed that the ATP energy coupling is of an indirect nature through some intermediate other than ATP.

Studying the energy expenditure by active transport mechanisms, Heinz and Patlak (112), calculated that the energy involved in the transport of glycine was 1890 $\pm$ 120 cal/mol. Since the value of the osmotic work due to the distribution of K^+ across the membrane was 1810 cal/mol., they suggested that possibly the K^+ gradient across the membrane drove the transport of glycine. The use of cyanide and 2-deoxyglucose to inhibit transport and exchange fluxes in Ehrlich cells has suggested that the linkage between the carrier system and energy metabolism was such as to either increase the rate of outward movement of free carrier or to increase the rate of dissociation of the complex at the inner portion of the membrane. The linkage was not to increase the rate of movement of complex across the membrane or to increase the rate of formation of the carrier-amino acid complex at the outer portion of the membrane (8).

A very interesting finding has been reported by Winkler and Wilson (113) who showed that in the case of β-galactoside transport in Escherichia coli, energy coupling increased the affinity constant of exit without affecting the affinity constant of entrance. They inferred from these studies that "energy coupling reduced the affinity of the carrier for its substrate on the inner surface of the plasma membrane." Such an observation still remains to be made with a mammalian transport system.

1.4 Amino Acid Transport in Different Tissues

(a) Kidney

As early as 1951 Dent and Rose (114) postulated that a single mechanism mediated the renal tubular reabsorption of lysine, arginine and cystine, and that cystinuria was a consequence of an inborn error in the normal transport process. Rosenberg et al (115) agreed that there was a common pathway for lysine, arginine and ornithine. Support for the inclusion of cystine in this grouping has come partly from the work of Stein (116) who found large quantities of L-lysine, L-arginine, L-ornithine and L-cystine in the urine of human cystinurics, and Robson and Rose (117) who showed that lysine infusions into normal patients caused increased renal clearances of cystine, arginine and ornithine, while lysine infusion into cystinurics caused little or no increase in renal excretion of cystine, arginine and ornithine. Other workers have disagreed with the inclusion of cystine. Segal et al (118), studying the effects of decreased pH and of the absence of oxygen and Na^+ on these amino acids, have concluded that cystine transport in kidney slices occurs by a system separate from that of the dibasic amino acids. This latter hypothesis is in agreement with the observation that the dibasic amino acids did not inhibit L-cystine transport nor did L-cystine inhibit transport of the dibasic amino acids (115). In kidney cortex slices from cystinuric patients (119), cystine uptake was normal in the presence of a defect in lysine and arginine accumulation in vivo. It was in 1966 that Schwartzman et al (120) showed that an increase of intracellular cysteine in lysine and ornithine preloaded kidney cells was due to an inhibition of cysteine

efflux by the dibasic amino acids. The difference in sensitivity to inhibitors suggested separate influx pathways for cystine and cysteine. They also concluded that cysteine was part of the dibasic amino acid transport system and that the mechanism of excessive urinary cystine excretion might be considered to be due to impaired cysteine efflux, rather than impaired cystine influx. It has recently been suggested that dibasic amino acid entry may involve more than one component, as demonstrated by Rosenberg et al (121) with L-lysine uptake. The two distinct transport systems for L-lysine were found in kidney cortex slices from both normal donors and from patients with cystinuria.

In addition to the dibasic system, there is a transport system in the renal tubule with preference for β-amino compounds (122) as well as a relatively specific mechanism for the reabsorption of acidic amino acids (123, 124). As far as transport of the neutral amino acids is concerned, evidence for a renal tubular system common to glycine, L-proline and L-hydroxyproline has been presented (125, 126), although L-proline and hydroxyproline have a greater affinity than glycine for transport in this system (126, 127). Support for this classification has come mainly from studies of diseases of the kidney. For instance, in Joseph's Syndrome, proline, hydroxyproline and glycine were excreted in very large quantities in the urine in the presence of a normal plasma concentration (128). Secondly, in Hartnup's disease (129) there is impaired transport of most of the neutral amino acids, but no impairment in absorption of the cystinuric group of amino acids or of proline, glycine and hydroxyproline (130). In familial hyperprolinemia (126, 131) there is

a specific hyperaminoaciduria comprising proline, hydroxyproline and glycine. Prolinuria occurred first, followed by an increased excretion of glycine and hydroxyproline in spite of a normal plasma concentration of these two compounds. To account for the excess of all three compounds in prolinuria, it was suggested that proline competed with these two amino acids for a common system in the renal tubule (126, 125, 132). Recent work (133) has demonstrated the presence of a mutant affecting renal transport of proline, hydroxyproline and glycine in man. The authors have proposed two different types of transport systems, one a common system and the other a system with lower capacity and greater specificity. Both of these types appeared to be controlled by different genes.

(B) Brain

Slices of brain cortex incubated in a suitable medium accumulated amino acids against a concentration gradient in vitro. Such accumulation was found for L-glutamic acid (39), D-glutamic acid (107), γ-aminobutyric acid (134), glycine (135, 108), 5-hydroxytryptophan (136), the L-isomers of histidine, proline, arginine, lysine, ornithine and methionine (137), and tyrosine (138, 139).

Histidine was concentrated to a greater extent in brain than in rat intestine, testis, kidney, spleen, liver, heart muscle, skeletal muscle or erythrocytes, whereas the brain took up L-tyrosine to a lesser extent than did intestinal mucosa, but to greater extent than did the remaining tissues (140). The presence of L- or D-acidic amino acids caused considerable inhibition of histidine uptake in brain, but had no effect

in intestinal mucosa, testis or spleen (141). In contrast, the L-isomer caused no inhibition and the D-isomer moderate inhibition in the kidney. Neame (141) concluded that the transport system for acidic amino acids in brain was less specific than that of other tissues. Many other amino acids also inhibited the uptake of L-histidine in rat brain slices (142). In addition, the brain had the capacity to transport histamine as well as several other imidazole derivatives (143).

The accumulation of L- and D-glutamic acid into brain cells was accompanied by K^+ movement and swelling of the cells, while in the case of GABA or β-alanine accumulation there was no change in the distribution of electrolytes and therefore no indication of cell swelling (77). These authors also showed that there was competitive inhibition between GABA and β-alanine, and between D-glutamate and L-aspartate.

Although most studies have been done with adult brain, Lahiri and Lajtha (76) showed that there were differences between the brain of adult and newborn animals. They found that adult brain slices accumulated L- and D-leucine and aminoisobutyrate to a greater extent, and L- and D-lysine to a lesser extent than newborn brain slices.

Yoshida et al (144) postulated that a specific site (called the L-DOPA site) combines with L-DOPA, phenylalanine and tyrosine in brain cortex slices of adult guinea pig. They concluded that since glutamate had no inhibitory effect on L-DOPA uptake, there must be a specific site for this acidic amino acid which differs from the L-DOPA carrier system. Neame and Smith (75) found that in the case of alanine uptake, the concen-

tration ratio obtained with the L-isomer after a short period of incubation was higher, but after a longer period was lower than that obtained with the D-isomer. This was explained as being due to the L-isomer moving out of the tissue faster than the D-isomer. In rat brain slices Nakamura (145) found that although both L- and D-methionine inhibited the uptake of L- and D-histidine, the uptake of DL-methionine was stimulated by L-histidine. In this latter situation, there was exchange diffusion between these two amino acids when L-histidine was present intracellularly and DL-methionine was present extracellularly. Abadom and Scholefield (146) concluded that there were several transport systems operating in rat brain cortex slices, each with its own specificity. A comparison of the specificities of the glycine transport system of brain and Ehrlich cells indicated that these two systems were quite different.

Recently Blasberg and Lajtha (72) have examined the substrate specificity of steady-state amino acid transport in mouse brain slices and have concluded that there are at least six different transport sites. The amino acids using these sites were classified as: small neutral, large neutral, small basic, large basic, acidic and GABA. They further agreed that each amino acid had an affinity for more than one transport site, i.e. a high affinity for its primary site of transport and a lower affinity for a secondary site of entry. Levi et al (147) showed that the intracellular presence of various amino acids inhibited or increased the exit rate of AIB, leucine, lysine and L-glutamate from mouse brain slices. They concluded that the amino acid exit, as well as the uptake,

was mainly a carrier-mediated process. Since the substrate pattern of amino acid exit was quite similar to the steady-state accumulation data, it was suggested that probably more than one carrier was involved.

(C) Ehrlich Ascites Cells

In the Ehrlich ascites cell there is considerable interaction between amino acids during the transport process (66, 148). Other experiments (70) with S-ethylcysteine and glycine revealed different optimum temperatures of uptake and different inhibitory effects of other amino acids. Based on these results, the authors suggested that "a variety of carriers exist in Ehrlich ascites cells that are responsible for concentrative uptakes of the amino acids."

Oxender and Christensen (67, 74) suggested that there were two types of mediating systems for the transport of neutral amino acids into the Ehrlich cell. One was termed the alanine-preferring system (A) and the other the leucine-preferring system (L). The former group included the amino acids alanine, glycine, serine, threonine, proline, asparagine, glutamine and methionine, while the latter group was comprised of leucine, isoleucine, valine, phenylalanine and L-methionine. The overlap between these two groups was very extensive, so that all, with the possible exception of glycine, might be represented in both groups. The alanine-preferring system (A) was shown to discriminate against branched hydrocarbon side chains, to have a high Q_{10} for the transport process, to have a high steady-state distribution ratio of amino acids, to operate

largely for entry and minimally if at all for exchange, and to be most sensitive to changes in pH or in the distribution of ions. The leucine-preferring system (L), on the other hand, showed much greater exchange diffusion, a lower sensitivity to pH or alkali-metal ion changes, a smaller Q_{10} than the (A) system, an increased affinity as the length and bulk of the side chain was increased, and a steady-state amino acid distribution ratio lower than that of amino acids using the alanine-preferring system (A).

Substitution of a single N-methyl group did not decrease the reactivity of amino acids with the "A" group, but largely eliminated reactivity with the "L" group and a lysine accepting system (92). The authors used the different effects of N-methylation (a) to eliminate the dominate uptake of phenylalanine by the L-system, so that its slower uptake by the A-system could be detected, (b) to synthesize N-methyl amino acids which could be used as inhibitors to eliminate the dominant migration of alanine by the "A" system in order to uncover other minor routes of migration. Their data indicated that "the interaction between neutral amino acids was based largely on their mutual sharing of these routes".

Jacquez and Sherman (8) have reported considerable exchange uptake in cells preloaded with glycine, L-proline and L-alanine, which is in contrast to the work of Oxender and Christensen who found very little stimulation of exchange diffusion via the A-system (67, 74). The former authors concluded that there was no reason to postulate the existence of two carriers, that in fact one could "relate differences among the two groups of amino acids as due to differences in affinities for one carrier

and to differences in the ease with which one amino acid displaces another from the carrier amino acid complex" (8).

In 1964 Christensen (149) proposed a lysine accepting system which served especially for entry of diamino acids and which differed from the (L) and (A) systems previously described in tolerating a positively charged side chain, although it retained a role in the transport of neutral amino acids. Thus the diamino acid entered by a system which served only as an additional minor route for the neutral amino acids. The mutual inhibition between cationic and neutral amino acids thus appeared to be limited almost entirely to the new system, which accepted amino acids whether or not their side-chains carried a positive change. The transport of lysine was greatly suppressed by a sufficient concentration of almost any neutral amino acid, whereas lysine was able to eliminate only a clearly defined portion of the uptake of neutral amino acids. The major portion of the entry of these neutral amino acid remained fully sensitive to either phenylalanine or AIB even in the presence of 50 mM lysine, indicating that entry by the A or L system had remained essentially intact. Furthermore, the mediated uptake of L-lysine (150) was composed of (a) a portion of about 60% which was eliminated by an excess of phenylalanine, (b) a second minor portion eliminated by various neutral amino acids, but not by phenylalanine and (c) another minor saturable portion which could not be inhibited by neutral amino acids and whose rate was doubled in a Na^+-free medium. Christensen and Liang have referred to (a) and (b) as the first and second lysine-accepting systems respectively, and to (c) as the lysine-preferring system.

Several other transport systems have also been proposed. For instance, there is a system operating for the uptake of α,α -diethylglycine which has a higher uptake at pH 5.0 than at pH 7.4 (86) and with respect to alkali-metal ion sensitivity, resembles the amino acids of the "A" system (67). Another system involves the uptake of β-alanine which at ordinary concentrations occurs by a distinct system with which the α-amino acids are also reactive, although one that participates little, if at all, in their total uptake (90). Kromphardt (151) showed that taurine, also concentrated in the Ehrlich cell, was hardly affected by α-amino acids, whereas β-alanine inhibited considerably and competitively. He concluded that taurine was transported by a mechanism which differed specifically from the one for the neutral α-amino acid, while β-alanine appeared to be transported by the mechanism serving taurine and the neutral α-amino acids.

The transport of ^{14}C-glutamate has recently been investigated by Heinz et al (152, 153) who showed that it was accumulated to a final ratio of between 1.5 and 1.6 after 1-2 hours. Despite a high transport potential, the intracellular accumulation remained low because it was limited by cellular metabolic utilization. They concluded that this amino acid, which had a higher uptake at acidic pH than at basic pH, was transported by a mechanism separate from that of the neutral amino acids.

(D) Intestine

As early as 1937, Hober and Hober (27) suggested that an active

transport process was involved in amino acid absorption in the intestine, since they found the rates of absorption of certain amino acids were too rapid to be accounted for without such a process. Subsequent in vivo work (26) showed that the presence of an excess of one amino acid could inhibit the intestinal absorption of another amino acid. This was followed by a number of studies which indicated that several amino acids could be accumulated against a concentration gradient in vitro. These included ACPC and α AIB (154, 155), L-cystine and L-cysteine (156), methionine (157, 158), L-histidine (159), L-monoiodotyrosine (160), as well as several other amino acids (161, 162).

When a racemic amino acid solution was introduced into a loop of rat small gut, the rate of disappearance of the L-isomer was greater than the D-isomer (163). Since then, several in vitro studies have also dealt with this particular aspect of the transport problem. Wiseman (164) measured the amount of amino acid passing through the gut wall by circulating the mixture through the lumen of isolated sections of gut, while the serosal surface was bathed in a similar solution. After one hour the concentration of the L-form decreased inside the lumen and increased in the outer fluid. In contrast, the concentration of the D-form remained constant in both the inner and outer fluids. Using isolated rat intestine, Agar et al (101) noted that the L-but not the D-form of histidine and phenylalanine was accumulated against a concentration gradient. Wiseman (102) found that the L-isomers of alanine, phenylalanine, methionine, histidine and isoleucine could produce concentration gradients, but that the transport of the D-isomers against a gradient could not be obtained.

Furthermore, the L-isomers of histidine and alanine were absorbed at a more rapid rate than the respective D-isomers by the isolated surviving guinea pig intestine (103), while the uptake of L-histidine was inhibited by L-but not D-amino acids (104). Finch and Hird (105) concluded that "studies with D-amino acids suggest they may combine with the site for uptake of L-amino acids, but have a lower affinity for it".

Several investigators have looked at the specificity of amino acid carriers in the intestinal transfer process. Since proline and glycine were good inhibitors of each other, Evered and Randall (165) agreed that "it seems likely that glycine and proline have a fundamental and primitive transport pathway...". The results of Newey and Smyth (166) suggested that there were probably at least two carrier systems for glycine, one of which was more susceptible to proline competititon and the other methionine competition. Based on other inhibition experiments, these same authors (167) concluded that in the intestine there was a carrier system common to glycine, proline and methionine, and an additional carrier for glycine and proline. Employing hamster everted intestinal sacs, Spencer and Brody (99) studied the effect of Na^+ lack, temperature increase, etc. on the transport of cyclic and non-cyclic amino acids. Although each compound inhibited the intestinal transport of the other, it was not possible to distinguish between different transport systems on the basis of the criteria used. More recent work by Munck (168) has shown the existence of a separate transport mechanism for imino acids which is also used by glycine, betaine, leucine and alanine. Munck pointed out that a demonstration of inhibition of glycine uptake by imino acids is complicated

by the affinity of glycine for two transport mechanisms, that of the neutral amino acids in addition to that of the imino acids.

Studies on interaction between different groups of amino acids have shown that the basic amino acids have little or no inhibitory effect on the neutral amino acid transport of L-histidine and mono-iodotyrosine (160, 161, 104). Hagihira et al (169) observed that the maximal rates of transport of the basic amino acids were 1/10 to 1/20 the rates of transport of some neutral amino acids (161). They concluded that intestinal epithelial cells possessed a "basic" amino acid transport system similar to that found in the proximal tubules of the kidney (169). Further work by Larsen et al (170) suggested that there were three separate amino acid transport systems in intestine since, although they noted a small degree of competition between the neutral and dibasic amino acids, there was no competition by amino acids of either of these two groups for the system which transported the N-methyl-substituted amino acids. The degree of neutral and dibasic interaction is illustrated by the neutral amino acid tryptophan which is a substrate for both the diamino acid and neutral amino acid carriers (171).

Recent in vitro work (172) has indicated that the transport of alanine and glycine could be inhibited by both galactose and fructose, although the inhibition of alanine transport by galactose was non-competitive. The findings of Bingham et al (173) were in favour of the view that sugars which were actively transferred, but not metabolized, inhibited amino acid transport. Since D-galactose and L-arginine were par-

·tially competitive inhibitors of neutral amino acid transport, Alvarado (174) has suggested that "all may share a common, polyfunctional carrier in which a series of separate binding sites, one for sugars, neutral amino acids, basic amino acids and Na^+ are joined together, as in a mosaic." All sugar interaction with amino acids need not be inhibitory as shown by glucose (actively transported and metabolized) stimulation of proline transfer, although no effect was observed on the transport of methionine into intestinal preparations (173, 175).

(E) Red Blood Cell

Christensen et al (176) showed that there was a concentrative uptake of alanine by duck erythrocytes and of glycine, L-alanine and L-glutamic acid by human erythrocytes. DL-Alanine as well as L-alanine were taken up by duck erythrocytes, while human erythrocytes apparently did not take up the D-form. They also found that the concentrative activity of erythrocytes was relatively insensitive to inhibitors of respiratory metabolism. Although rabbit reticulocytes had higher intracellular glycine levels than plasma, the mature rabbit erythrocyte did not possess any concentrative ability for this amino acid (177). In contrast to mature erythrocytes, the concentrative mechanism in the reticulocytes was inhibited by high concentrations of cyanide, DNP and arsenate. Subsequent work (178) on the migration of amino acids across the membrane of the human erythrocyte, indicated that there were three modes of uptake: (a) a mediated transport used by almost all the neutral amino acids, preferring long chain amino acids and easily saturable by these

compounds, (b) a low capacity, saturable uptake for glycine and alanine, and (c) an apparently non-saturable component limited to amino acids with large hydrocarbon side chains. Vidaver et al (179), examining the specificity of amino acid entry routes in pigeon erythrocytes, found that the glycine entry route was highly specific and evidently quite distinct from the L-alanine route.

Winter and Christensen (84) in 1965 concluded that in the rabbit reticulocyte there were at least three transport systems for amino acids similar to the A-group of the Ehrlich cell (67), as well as a leucine-preferring system. Only the latter was retained upon maturation of the cell. Alanine uptake by the reticulocytes was much more sensitive to cation changes than the erythrocytes, while countertransport of L-alanine was demonstrable in reticulocytes, but absent upon maturation to the erythrocyte stage (84).

(F) Pancreas

The in vitro accumulation of amino acids by pancreas has been studied only recently and so the available data is rather limited. Bégin and Scholefield (46) in 1964 demonstrated for the first time in vitro that amino acids such as glycine and ACPC could be accumulated against a concentration gradient in mouse pancreas and that such a system was affected by anaerobiosis, DNP, ouabain and variations in the Na^+ and K^+ content of the external medium. An examination of the specificity of the carrier systems on the basis of the ABC test showed that at least three separate sites were involved in the transport mechanism (48). One site

was utilized by L-valine, ACPC and MeACPC, the second by glycine and the third served for L-methionine and L-ethionine entry. Begin and Scholefield concluded that "each is transported into mouse pancreas as a result of combination with only one carrier site and since the amino acids have affinities for other sites at which they are not transported, there is competitive inhibition of the transport of substrates which are translocated as a result of combination with these sites". Further studies (47) provided a fourth site for L-proline uptake, since Lineweaver-Burk analysis indicated that proline was transported only after two molecules had combined with adjacent sites on the surface of the carrier.

By means of perfusion of isolated rat pancreas, Sprecker and Bennett (180) found that, although the normal pancreas was capable of concentrating L-valine, glycine and AIB, the pancreas of hyphysectomized and adrenalectomized animals had a 50% reduction in amino acid accumulation without a significant change in the incorporation into protein. This accumulation was partially blocked with 5×10^{-4} DNP and the incorporation was completely blocked with puromycin without affecting the accumulation. They suggested that hormonal stimuli was necessary for the transfer of the amino acids without having a direct effect on the incorporation.

1.5 The Role of Ions

Many studies have stressed the importance of an altered ionic medium on the carrier-mediated uptake of amino acids. In 1952 it was

found that incubation of Ehrlich ascites carcinoma cells in a glycine containing medium resulted in both an accumulation of the amino acid and a loss of K^+ from the cells (63). Subsequent work (181) showed a decreased glycine uptake if either the extracellular or intracellular K^+ level were lowered. Restoration of the intracellular K^+ concentration brought about a normal glycine accumulation by the Ehrlich cell. Based on the observations, a mechanism was proposed whereby K^+ efflux drove glycine influx via exchange diffusion of extracellular amino acid for intracellular K^+.

More recent work has tended towards rejection of this hypothesis. Studying the relationship between the maintenance of glycine gradients and steady-state K^+ fluxes, Hempling (182) noted that incubation in a low glycine medium resulted in both an increased K^+ efflux and influx. He argued that if the above hypothesis was correct, then there must be a separate route for K^+ influx which also increases when glycine is accumulated. Since cells also showed an increased water content during amino acid accumulation, he suggested that perhaps "cell swelling may also be a factor which contributes to the increase in the K^+ fluxes observed." In short-term experiments, reduction of cellular K^+ by pre-incubation with K^+-free solutions increased the K^+ influx, but had no effect on the uptake of glycine (83). Additional evidence against the role of K^+ has come from the work of Hempling and Hare (183) who found that glycine had a greater affinity than K^+ for the carrier at both the outer and inner portions of the membrane. If exchange diffusion accounted for glycine uptake, then glycine should have a greater affinity for the

carrier at the outer membrane and a lower affinity than K^+ intracellularly. Also against this hypothesis was that in order for K^+ to displace glycine from the carrier at the inner portion of the membrane, the cell must contain 25 times more K^+ than glycine (183). In addition, since the energy available from K^+ efflux was not sufficient to pump glycine inward, K^+ efflux could not be responsible for glycine influx (183). These authors proposed an alternative hypothesis which suggested, instead, that glycine transport facilitated K^+ transport.

Since Ricklis and Quastel (184) first showed that the Na^+ ion was required for sugar transport in the intestine, many studies have been concerned with the apparent involvement between Na^+ and sugar movement. Bihler and Crane (185) confirmed that the active transport of sugars was absolutely dependent upon the presence of Na^+ in the medium, whereas the absence of external Na^+ had no significant effect on the influx of compounds which were not actively transported. Subsequent work (186) indicated that with certain sugars there was both a Na^+-dependent, energy independent entrance and a Na^+-dependent, energy dependent accumulation against a concentration gradient.

Examination of a possible link between active transport of ions and non-ionic compounds showed that the transport of several amino acids was strongly inhibited if Na^+ was absent from the solution bathing the mucosal surface of the intestine (187). Furthermore, Csaky concluded that for transport of sugars to occur, the Na^+ ion must be on the membrane of the mucosal epithelium facing the lumen (2). In this view it was not the carrier which required Na^+ for functioning, but that the cation was essential

for the non-specific part of the pump (2, 188). I.e. Na^+ required for coupling of metabolic energy to the carrier system for non-electrolytes. Subsequent studies tended to disprove this latter hypothesis of Csaky. It was found that a change in the Na^+ concentration of the external medium altered the apparent K_m value for uptake, but did not affect the V_{max} (189, 190). The hypothesis of Crane and his co-workers consisted of a mobile carrier with two binding sites; one for sugar substrates and the other for monovalent cations, so that the Na^+ moved across the membrane barrier with the substrate. When the carrier was loaded with Na^+, the K_m was low, while a K^+ loaded carrier was responsible for a high K_m value. The intermediate K_m values observed depended upon the relative proportions of these two species at any given time. Lyon and Crane (191), examining transmural potential differences across intestine membranes, have proposed two kinetic models based on their results. One is in the rat where there is a large degree of interaction between the ion and sugar binding sites of the carrier and the other is in the rabbit where the interaction between these sites is minimal.

The net movement of sugar into the intestine has been viewed as a consequence of a "downhill" gradient of Na^+ into the cell maintained by the operation of an outwardly-directed, energy dependent Na^+ pump. As a test of this hypothesis Crane (192) found that when intestinal villi preloaded with a sugar were transferred to a medium containing no Na^+ (thereby reversing the Na^+ gradient), the sugar moved "uphill" out of the cells. This latter observation offered additional evidence that the

"downhill" Na^+ gradient into the cell was responsible for an "uphill" sugar movement in the same direction.

In pigeon red cells the entry of glycine involved two components; one Na^+-independent and the other Na^+-dependent (193). The influx of this amino acid required two Na^+ ions as co-factors (193, 194) and alteration of the external Na^+ concentration affected the K_m rather than the V_{max}, suggesting an effect of the ion on glycine binding to the carrier (193). Cells which were lysed and then restored (195) were capable of accumulating amino acids only if a Na^+ gradient existed and reversal of this gradient (high Na^+ inside, high K^+ outside) resulted in glycine being pumped out of the cells. This supported the hypothesis that the Na^+ gradient furnished the energy for the transport of glycine. Wheeler et al (196) noted that in pigeon erythrocytes and rabbit reticulocytes, a change in the external Na^+ concentration resulted in both an altered V_{max} and K_m value. Subsequent work dealing with the role of Na^+ in amino acid transport into rabbit red cells, confirmed this change in the V_{max} (197). In this system there was no saturable uptake of glycine or alanine in the absence of Na^+. In the case of alanine uptake, one Na^+ ion appeared to function as co-factor; whereas with glycine entry, two Na^+ ions seemed to be essential.

Several studies have examined the relationship between amino acid transport and the Na^+ - K^+ pump in human leucocytes (198, 199). It was found that AIB, glycine, proline, and alanine required Na^+ for transport, were inhibited by ouabain and stimulated by Ca^{++}. Amino acids such as ACPC, arginine, lysine, histidine, methionine were not Na^+-dependent,

not inhibited by ouabain and not stimulated by Ca^{++}. The Na^+ and Ca^{++} effects on the steady-state levels were due to a stimulation of influx, the efflux being unaffected (199).

Inui and Christensen (200) have reported that the uptake of L-methionine, AIB and MeAIB in the Ehrlich ascites cell occurs by at least two transport systems, one sensitive and the other insensitive to the presence of Na^+. With AIB and MeAIB the Na^+ insensitive uptake is not concentrative, whereas with L-methionine the Na^+ insensitive uptake is concentrative. These three amino acids showed both an altered K_m and V_{max} value with changes in the external Na^+ concentration, thus in agreement with the findings reported for rabbit red cells (196, 197).

Removal of Na^+ from the external medium resulted in a lack of ability of rat kidney cortex slices to concentrate AIB or glycine, whereas lysine and histidine were still accumulated, although to a considerably lesser extent (201). The entry of these latter amino acids appeared to be mediated by two mechanisms; one Na^+-dependent and ouabain sensitive and the other Na^+-independent and ouabain insensitive. Segal et al (118) found that in a Na^+-free medium the initial rate of lysine accumulation was not affected, although the steady-state concentration was reduced. With cystine and arginine the initial rate was inhibited by Na^+ lack and there was no evidence of a concentration gradient at the steady-state. Examination of the effect of Na^+ on the kinetics of AIB transport (202) showed that a lowered external Na^+ concentration resulted in a diminished

initial and steady-state influx. Reduction of the external Na^+ concentration to less than the intracellular concentration, caused an increase in both steady-state and initial efflux. The decrease in external Na^+ concentration affected the K_m value, but did not alter the V_{max}, thereby in agreement with the findings of Crane and Vidaver (189, 190, 193) and in contrast to the observations of Inui and Christensen (200).

In addition to the effect of Na^+ on amino acid transport in the kidney, recent studies have examined the role of other ions. Brown and Michael (203) have shown that the net accumulation of inorganic phosphate in rat kidney cortex slices is inhibited by several neutral, aromatic and polar L- or D-amino acids. The same authors (204) noted that in renal cortical slices from parathyroidectomized rats, the net accumulation of several amino acids was inhibited by a decrease in the concentration of Ca^{++} or inorganic phosphate.

Several other systems have been very briefly studied with respect to the relationship between the Na^+ ion and amino acid transport. Parrish and Kipnis (205) observed that although AIB entered the rat diaphragm via a carrier-mediated transport system in the complete absence of Na^+, it was not concentrated intracellularly. The influx of AIB was twice as rapid in the presence of Na^+ than in its absence, whereas the efflux was not changed by Na^+. Although the Na^+ pump is linked to amino acid accumulation, the exact nature of this relationship is not completely understood, since a 1×10^{-5} M concentration of ouabain had no significant effect on AIB entry even though it markedly

depressed Na^+ pump activity (205).

Preliminary work on amino acid transport in bone (82) has shown that glycine and AIB were still accumulated by a mediated process in the absence of Na^+, but that L-proline and hydroxyproline entry was non-mediated resembling simple diffusion very closely. In the presence of Na^+ the amino acids, glycine, AIB, L-proline and hydroxyproline were mutually inhibitory, whereas in the absence of Na^+ glycine and alanine still inhibited AIB uptake, while proline and hydroxyproline were without effect. Glycine, AIB and hydroxyproline inhibited L-proline uptake only in the presence of external Na^+ and not when it was omitted from the incubation medium.

In the rabbit eye lens (206) the transport systems for AIB, neutral, basic and acidic amino acids involve both a Na^+-dependent and a Na^+-independent component. In the case of AIB entry one Na^+ per amino acid appeared to function as co-factor, while a change in the external Na^+ concentration affected the K_m value but not the V_{max}. In this sytem Na^+ had no effect either on amino acid efflux nor on the diffusion constant for entry.

The possibility of a linkage of the Na^+ - K^+ pump to amino acid transport has been strengthened by the observations that ouabain inhibition of the Na^+ - K^+ stimulated ATPase also results in a decreased amino acid accumulation in both single cell preparations and in tissues (46, 207). The linkage of an ionic pump mechanism to substrate movement has received further attention from the work of Milner and Hales (208) who reported

that both ouabain addition and K^+ omission from the external medium, stimulated insulin secretion from the β-cells of rabbit pancreas.

Homo-exchange diffusion of L-methionine in Ehrlich ascites cells appeared to be independent of the presence of either Na^+ or K^+ (209). In contrast to these findings, the countertransport process for alanine (in or out) in rabbit red cells was dependent upon the presence of Na^+; whereas valine, which has a Na^+-independent uptake, was not affected by Na^+ ions (197). This apparent discrepancy as to whether or not ions are required for the exchange diffusion process as well as the transport process, may possibly be due either to major differences between the above two systems or to a cation requirement with only certain amino acids. Perhaps in the Ehrlich cell, amino acids, other than L-methionine, may show a Na^+-dependence for the exchange diffusion process.

1.6 Exchange Diffusion

Although the term exchange diffusion has been used with respect to an additional mode of entry for sugars and amino acids, it was originally introduced to explain ion movements across the membrane barrier (210, 211). In this process the carrier-ion complex moved from the inner to the outer portion of the membrane where exchange of the bound ion for a free extracellular one occurred. This new complex then moved inward where another exchange of ionic species took place. The transport and exchange diffusion processes were thought to be mediated through the same carrier system, with exchange resulting in no net transport, since as Ussing (210) had argued "the same number of ions will be carried

from left to right as from right to left".

In Ehrlich ascites carcinoma cells, preincubation of the cells with unlabelled glycine resulted in an increased influx of the radioactive amino acid (64). This finding was subsequently confirmed by Heinz (212) in 1957 who concluded that "the kinetics of this exchange are consistent with the assumption that most of the accumulated glycine is free...". From a comparison of the relative rates of transport and exchange diffusion in this system, Heinz and Mariani (213) calculated that the maximal transport rate was 10 μmoles per gm (dry weight)/minute, while the maximal rate of influx was 57 μmoles per gm/minute. Therefore exchange diffusion was more than five times as rapid as active transport. In addition, Heinz and Walsh (7) found that the efflux of amino acid increased proportionally if the intracellular glycine concentration was raised to 50 mM. This preloading with glycine resulted in an increased influx coefficient for glycine without affecting the efflux coefficient. They proposed a schematic model to explain the process of active transport and exchange diffusion occurring simultaneously. As adopted from ion exchange diffusion, the carrier-amino acid complex which moves from the outer to inner portion of the membrane represents both extracellular substrate bound to the carrier via the transport process and extracellular amino acid attached via an exchange for the amino acid which the carrier had picked up intracellularly. Once in the inner portion of the membrane, the carrier-amino acid complex unloads only part of its amino acid, while the rest is exchanged with intracellular amino acid. In the former case the free carrier instantly undergoes

catalytic "inactivation" to prevent outward movement of an unloaded, active carrier (this inactivation step is essential for accumulation to occur). In the second situation the new carrier-amino acid complex formed intracellularly can now exchange with more extracellular amino acid once again.

A study of the effects of lipotropic agents on the transport and exchange diffusion processes led Johnstone and Quastel (214, 215) to conclude that the same carriers were involved in both processes and that "lipotropic agents are inhibitory because of their association with lipid components in the cell membrane controlling transport". Jacquez and Sherman (8) found that the transport fluxes, but not exchange fluxes were inhibited by a number of metabolic inhibitors. Their observations suggested that the linkage between the carrier system and energy metabolism was such as to increase the rate of outward movement of free carrier or to increase the rate of dissociation of carrier-amino acid complex at the inner surface of the membrane. The linkage was not to increase the rate of movement of carrier-amino acid complex across the membrane or to increase the rate of formation of the carrier-amino acid complex at the outer surface.

Examining the structural specificity of the glycine transport system in this cell, Paine and Heinz (148) noted that generally compounds which failed to inhibit glycine influx were also unable to exchange with glycine, although L-alanine which was an excellent inhibitor of glycine influx, failed to increase glycine influx if previously accumulated intracellularly. This effect was later studied in more detail by Oxender

and Christensen (67, 74). The authors concluded that amino acids could be divided into two separate groups on the basis of their ability to exchange. Compounds such as valine, ACPC and L-methionine were very excellent exchangers, while the group comprising AIB, glycine and alanine were very poor as exchanging amino acids.

Upon addition of several amino acids such as ethionine, etc. to Ehrlich ascites cells in equilibrium with DL-methionine-S^{35}, there was an extensive loss of radioactivity followed by a slow recovery of the lost radioactivity (73). This was explained as being due to a rapid exchange diffusion, followed by a rate of re-entry determined by the relative affinities of the two amino acids. Since exchange diffusion involves the movement of a solute across a membrane in strict mole-to-mole exchange for a similar solute moving in the opposite direction, Johnstone and Scholefield (73) examined the stoichiometry of the exchange process in this system. They found that the ratio of the rate of movement of methionine into the cells compared with the rate of movement of ethionine out was 1:1.28. The movement in the opposite direction (ethionine in and methionine out) was 1:1.20. These values were sufficiently close enough to a mole-to-mole exchange ratio to satisfy the criteria set forth for true exchange diffusion.

Oxender and Christensen (67) obtained affinity constants of several amino acids for the exchange reaction in the Ehrlich cell by preloading the cells with an amino acid and then stimulating efflux by varying the concentration of amino acid in the external medium. The

amino acids valine and methionine had identical affinity constants for both the transport and exchange diffusion processes, causing the authors to conclude that these amino acids "saturated the driving of counterflow when their concentration are brought to levels that also saturate their own migrations". However, other amino acids such as AIB, alanine and glycine had K_m values for exchange diffusion which were many fold greater than those for transport. Oxender and Christensen concluded that "since the small driving of the outward migration by the amino acids in the external position is nearly linear up to 70 mM, whereas the K_m values for their uptake are 0.5 to 0.6 mM, in driving exodus they must be reacting with a chemical site other than the one by which they enter the cell".

Exchange diffusion has been studied in a number of other systems, although not as extensively as in the Ehrlich ascites cell. In vivo experiments with brain (216) showed that lysine-C^{14} administered intravenously or intraperitoneally entered the circulating blood and exchanged rapidly with the free lysine of the brain. Subsequent work (217) demonstrated that the exchange rates of amino acids were stimulated by increasing the amino acid concentration in the brain. Nakamura (145) showed in vitro exchange diffusion between histidine and DL-methionine in rat brain slices, although exchange was seen only when histidine was present intracellularly. The ratio of the loss of L-histidine to the entry of DL-methionine was calculated by Nakamura (145) to be 1:1.09. In this instance only the total stimulated values, which included a quite considerable transport component, were used. Substraction of this

transport portion would result in a ratio approaching 1:2. Further, there was no effect of ouabain on exchange diffusion in rat brain cortex slices (218), which is in agreement with the observation that ions are not required for the exchange diffusion of amino acids in Ehrlich ascites cells (209).

Levi et al (147), examining the substrate specificity of cerebral amino acid exit in vitro, noted that with AIB intracellularly, the greatest effect was obtained when AIB itself was present extracellularly. Significant increases in AIB efflux were also obtained in the presence of glycine, alanine and ACPC. Leucine when present intracellularly, was a very poor exchanger, while in the case of L-lysine intracellularly, the large basic amino acids had the greatest effect. This was in contrast to the neutral and acidic amino acids which were quite poor. In the case of glutamate intracellularly, there was almost negligible exchange with external neutral and basic amino acids, but quite good exchange with acidic amino acids.

Using everted sacs of rat intestine, Mochida et al (219) showed in vitro that L-histidine and L-methionine exchanged through the intestinal wall. Their results suggested that exchange diffusion occurred from different compartments of the preparation at varying rates. Preloading of the small intestine with leucine resulted in a stimulated uptake of lysine (220), while tryptophan entry was increased by methionine, leucine and sarcosine (171). Since the stimulation of lysine uptake by leucine was obtained whether the intestine was preloaded with leucine or whether leucine and lysine were present together on the mucosal surface, Munck (221) explained this phenomena in terms of the combined function of the

transport mechanisms of the neutral and dibasic amino acids. He concluded that "of the two transport mechanisms, that of the neutral amino acids created and maintained, on the serosal side.... a concentration of leucine sufficient to exert a counterflow effect on the transport of lysine by the transport mechanism of the diamino acids".

In rat kidney cortex slices, lysine, arginine, ornithine and diaminobutyric acid participated in both auto-exchange and hetero-exchange diffusion, whereas neither cystine nor cysteine exchanged with these dibasic amino acids (222). Preloading with lysine did not stimulate the uptake of histidine, phenylalanine or ACPC, while preloading with glycine, leucine or valine did not significantly increase the uptake of lysine. These same authors (223) reported that anaerobiosis and ouabain had no effect on exchange diffusion of the dibasic amino acids, and that a Na^+-dependence for exchange could be obtained. They also noted that the exchange uptake of lysine was not altered at reduced temperatures, while exchange diffusion of arginine was markedly inhibited at 21°C.

In the red blood cell there are membrane carriers to facilitate the equilibration of intracellular and extracellular substrate concentrations, without the expenditure of energy. Thus there is facilitated diffusion without the intracellular accumulation observed in active transport. Rosenberg and Wilbrandt (224), by equilibrating red blood cells with a penetrating sugar (C^{14}-glucose) and then adding a second penetrating sugar (unlabelled glucose or mannose), have demonstrated an uphill transport of glucose induced by counterflow. This latter process appears to be iden-

tical with the exchange diffusion mechanism shown in various mammalian systems. They concluded that "flow-induced uphill transport is a feature characteristic for mobile carrier systems only and is not to be expected in systems in which the substance is bound to a fixed membrane component, although such a system may yield identical transport kinetics". LeFevre and McGinniss (225) in 1960 confirmed these findings by first equilibrating cells with unlabelled glucose and then adding C^{14}-glucose to follow the entry of the tracer. They found that the speed of the tracer movement in relation to the speed of net uptake was about 50 to 100 times more rapid than that existing in a simple diffusion process. In addition, hetero-exchange diffusion between sugars is also possible since when glucose was added to cells equilibrated with sorbose, the intracellular sorbose moved out of the cell against a concentration gradient (226). Glucose exited very rapidly from the cell if D-galactose, D-mannose and D-xylose were present in the medium, whereas in the presence of D-fructose, L-sorbose and D-arabinose, the efflux of D-glucose was much slower (227).

A kinetic comparison of exchange transport with non-exchange transport of sugars in human erythrocytes showed that in the former case the V_{max} and K_m increased with rising temperature, whereas with non-exchange transport the V_{max} increased, while the K_m remained unaltered (228). The K_m value for exchange diffusion with galactose as the substrate was seven times higher than the K_m of the non-exchange transport. In addition, the activation energy for exchange transport was three times lower than that for non-exchange transport (228).

1.7 Objectives of the Present Work

The total movement of an amino acid into a cell can be regarded as the sum of three separate processes, namely simple diffusion, active transport and when demonstrable, exchange diffusion. The present work will, first of all, examine the characteristics of and the factors influencing both Na^+-dependent and Na^+-independent active transport in mouse pancreas. Secondly, the tissue will be studied with the objective being (a) to provide evidence for pancreatic exchange diffusion, (b) to examine the factors affecting and controlling such a process, and (c) to indicate the possible similarities and dissimilarities between this phenomenon and carrier-mediated active transport.

CHAPTER 2

MATERIALS AND METHODS

2.1 Amino Acids

Glycine-1-C^{14}, L-proline-C^{14}(U), L-lysine-C^{14}(U), and L-methionine-methyl-C^{14} were purchased from the Radiochemical Centre, Amersham, England. L-Tryptophan-3-C^{14}, α-aminoisobutyric-1-C^{14} acid, 1-amino-cyclopentane-1-carboxylic acid - carboxyl-C^{14} and γ-aminobutyric-1-C^{14} acid were obtained from the New England Nuclear Corporation, Boston, Massachusetts, U.S.A. Stock solutions were prepared by dissolving the radioactive amino acids in either 5 or 10 ml of distilled water.

The amino acid solutions used in these studies were prepared by diluting an aliquot of the stock solution and adding sufficient unlabelled amino acid to give a concentration of either 30 or 60 mM, with specific activities ranging from 50 cpm/mμM to 400 cpm/mμM. All solutions were kept frozen at -20°C and thawed out immediately prior to use, thereby preventing any bacterial growth or deterioration of the amino acids.

The unlabelled amino acids were purchased from Nutritional Biochemicals Corporation, Cleveland Ohio, and were used without further purification.

2.2 Animals

Male Swiss white mice weighing between 20 and 25 g were used throughout these studies. Results obtained with animals weighing in ex-

cess of 25 g did not differ significantly from those obtained with the 20-25 g mice.

2.3 Preparation of the Tissue

The mice were killed by cervical dislocation and an incision was made to the left of the mid-line on the ventral side. The pancreas and spleen were removed as a single unit, the spleen discarded and the pancreas placed on a Petri dish filled with crushed ice in order to keep the tissue at a temperature of 0-4°C prior to incubation. When spread out, the pancreas assumed a "horseshoe" shape and several thin strips were obtained by making incisions with small scissors along the sides of the "horseshoe". The amount of tissue used in each vessel consisted of several strips having a total weight of between 50 and 70 mg.

2.4 Incubation Conditions for Transport Studies

The strips of pancreas were incubated in a calcium-free Krebs-Ringer solution containing 145 mM NaCl, 5.8 mM KCl, 1.4 mM KH_2PO_4 and 1.4 mM $MgSO_4$ buffered with 10 mM sodium phosphate to a final pH of 7.4. For the Na^+-free incubations, the NaCl and sodium phosphate buffer were replaced by equimolar concentrations of choline chloride and tris chloride buffer respectively. The final volume in all cases was 3 ml. The vessels were gassed with 100% oxygen for 3 minutes and the incubation was carried out at 37°C in stoppered 25 ml Erlenmeyer flasks in a water-bath shaker. The length of the incubation varied from 30 to 90 minutes depending upon the time required for the individual amino acids to reach their steady-state levels.

2.5 Determination of Amino Acid Uptake

At the end of the incubation period, the flasks were removed from the water-bath shaker and placed in a tray containing crushed ice. The tissue was carefully removed with forceps and transferred to test tubes containing 8 ml of ice-cold saline. The samples were then centrifuged for approximately 40 seconds at 800 xg (in an International Equipment Company Clinical Centrifuge), the supernatant discarded and the sides of the tubes dried with tissue paper. The free amino acids were obtained by adding 3 ml of 5% TCA to each tube and then homogenizing with a motor driven apparatus. The samples were allowed to stand for 30 minutes, after which time they were centrifuged and an aliquot of the TCA soluble portion counted. In practice, standards of the radioactive amino acid solution were prepared and counted along with the samples in each experiment. In addition, at the end of the incubation period, mouse pancreas from other species and salivary glands of mice and rats (Chapter 3) were processed in exactly the same manner as mouse pancreas indicated above.

Counting was carried out using a series 3000 Packard Instrument Company liquid scintillation counter. The aliquots were counted in glass vials containing 10 ml of a scintillation mixture consisting of 5.0 gm 2,5-diphenyloxazole (PPO), 50 mg 1,4-bis-2-(4 methyl-5-phenyloxazolyl)-benzene (dimethyl-POPOP), and 80 gm naphthalene in 1 litre 1:1:1 (V:V:V) toluene:dioxane:ethanol. In all instances 0.2 mls of the TCA soluble extract was counted and any error due to quench variation was not encountered.

The counts per minute (cpm) thus obtained were corrected for background, multiplied by 15 (0.2 ml sample taken from 3 ml), multiplied by 1000/the weight of the tissue in mg (to express the results per gm wet weight), divided by 0.75[1] (to correct for the amount of water in the tissue) and finally divided by the specific activity of the amino acid (cpm/μmole) to obtain results expressed as μmoles of amino acid taken up per gram tissue water. For these studies it was also necessary to know the final concentration of amino acid in the medium. This was obtained by counting 0.5 ml of the external medium and a standard consisting of an aliquot of the working solution made up in Krebs-Ringer phosphate solution in a vial containing 10 ml of the scintillation mixture. All the uptake results quoted are the mean values of at least five separate determinations except in the case of the kinetic studies where a larger number of observations were used in order to insure greater accuracy.

2.6 Estimation of the $C^{14}O_2$ Produced

These experiments were carried out in 25 ml Erlenmeyer flasks in which 0.2 ml of Hydroxide of Hyamine 10-X had been placed in the center well. The reaction was terminated by injecting 0.2 ml of 30% TCA through the rubber stopper into the main compartment and allowing the incubation to proceed for another 15 minutes to insure the complete reaction of the $C^{14}O_2$ with the Hyamine. The contents of the center well were then removed and counted in 10 ml of scintillation mixture as previously described.

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1 This value was obtained by drying tissues to a constant weight and taking the dry weight/wet weight ratio.

2.7 Measurement of the Influx Portion of Exchange Diffusion

The strips of pancreas were incubated at 37°C in oxygen gassed 25 ml Erlenmeyer flasks in the presence and absence of a suitable concentration of non-radioactive amino acid. This resulted in a desired level of amino acid in the tissue. At the end of the incubation period, the amino acid "loaded" and "unloaded" tissues were removed with forceps, washed in 8 ml of ice-cold saline and freed from excess moisture by passing the strips along a portion of parafilm. The tissue was then transferred to a new oxygenated Krebs-Ringer phosphate medium containing a radioactive amino acid and the incubation was carried out at 15°C for a desired period of time. The uptake of radioactive amino acid by the "loaded" and "unloaded" tissues was then determined as previously described.

2.8 Measurement of the Efflux Portion of Exchange Diffusion

The preincubation was carried out at 37°C in 25 ml Erlenmeyer flasks all containing a labelled amino acid. The period of incubation ranged from 60 to 90 minutes depending upon the particular amino acid. This resulted in an exact level of radioactivity intracellularly. The tissue was then similarly removed, washed, freed from excess moisture and transferred to a new oxygenated medium in the presence and absence of unlabelled amino acids. This second incubation was carried out at 15°C using a large volume of external medium (9 ml) in order to minimize the possibility of reconcentration. In experiments where the initial concentration of radioactive amino acid in the tissue was greater than 10 mM,

the volume of the external medium was increased accordingly in order to maintain the same dilution factor for the exiting amino acid. The efflux of the labelled amino acid was followed by taking aliquots of the external medium at definite time intervals and counting as previously described.

The concentration of amino acid exiting from the pancreas was calculated as follows: the first 0.2 ml aliquot was multiplied by 45 to obtain the total cpm in the 9 ml of external medium. Subtraction of the amount of radioactivity in this aliquot from the total efflux gave the radioactivity (cpm) remaining in the incubation medium. Subsequently, another 0.2 ml sample of the medium was removed several minutes later and the radioactivity in this aliquot was determined. The total medium radioactivity (cpm) was then calculated by multiplying the cpm of the aliquot by 44 (since now the volume of external medium was 8.8 ml). The radioactivity which had exited in the second interval of time was obtained by subtraction of the cpm of the medium left after the first aliquot from the total medium cpm as determined by the second aliquot. Similarly the remaining medium radioactivity was again obtained by subtraction of the cpm removed in the second aliquot from the calculated total cpm of the medium. Then once again a third aliquot at a definite time will be taken from an external medium of 8.6 ml. By this method it was possible to calculate the total cpm leaving the tissue at specific time intervals. This figure was then multiplied by 1000/the weight of the tissue in mg, and divided by 0.75 (to correct for the amount of water in the tissue) and the specific activity of the amino

acid, in order to determine the concentration of this substrate in the tissue. Therefore the amount of substrate leaving the pancreas is expressed as μmoles of amino acid/g tissue water.

After the last aliquot the tissue was removed; washed, homogenized in 3 ml of 5% TCA, and the concentration of amino acid in the TCA soluble portion determined. This value was then added to the total amount of effluxed amino acid in order to determine the initial concentration of amino acid in the tissue prior to the start of the second incubation. Only when these totals were identical could a valid comparison be made between the relative effects of different external amino acids. All efflux values reported in Chapter 6 represent the mean of at least six determinations.

2.9 Chromatography

The TCA soluble extract was washed 5 times with 5 ml portions of ether to remove all of the TCA. The sample was evaporated to dryness and the residue was taken up in 0.1 ml of 95% ethanol. Portions of the extract were spotted on 10 in x 9 in sheets of Whatman No.1 filter paper and the chromatograms were run in an ascending fashion in sec-butanol: 90% formic acid:water (100:15:25) for 16 hours and in the second dimensions, tert-butanol:methyl-ethyl-ketone:ammonia:water (50:50:15:25) for another period of 16 hours. The chromatograms were exposed for 7 days on Kodak Blue Brand Medical X-ray film and compared against standards to determine whether there was more than one radioactive area. Radioactive scanning of the chromatogram before and after chromatography gave a value representing the percentage of amino acid still unchanged.

CHAPTER 3

THE PANCREAS-AN IN VITRO AMINO ACID TRANSPORTING TISSUE

Bégin and Scholefield (46-48) demonstrated *in vitro* that pancreas from normal Swiss white mice actively accumulated several amino acids against a concentration gradient. The present preliminary studies were concerned with (A) the uptake of amino acids by mouse pancreas under different conditions, (B) the concentrative ability of mouse pancreas compared to that of other experimental animals, and (C) its transport capacity relative to a normal tissue of similar structure.

3.1 Amino Acid Uptake in Swiss White Mice

ACPC, a non-metabolizable amino acid, was used as a model substrate in these studies. As can be seen in Table 1, Swiss white mice weighing 20 or 40 g, whether on a regular diet or fasted for 72 hours prior to experimentation, gave almost identical uptakes. However, amino acid accumulation in pancreas of newborn mice was approximately 32 per cent lower than that of the adult, while pancreas from adult mice bearing an Ehrlich ascites carcinoma tumor showed a much greater reduction in the concentrative process. An increased amino acid uptake could not be demonstrated as a result of alteration of the physiological state of the experimental animal.

3.2 Concentrative Ability in Various Species

In these studies (Table 2), several natural and unnatural amino

TABLE 1

ACPC-C^{14} Accumulation by Mouse Pancreas

Source of Pancreas	Uptake (μmoles/g tissue water)
20 G. Mouse	16.2
40 G. Mouse	16.0
Fasted Mouse (72 hours)	16.1
Mouse bearing an Ehrlich ascites tumor	6.1
Newborn Mouse (1 week)	10.9

The incubation was carried out at 37°C for 60 minutes with 2 mM ACPC - C^{14} in the external medium as described in "Materials and Methods". All values in this and subsequent tables represent the mean of at least five observations unless otherwise indicated.

TABLE 2

Comparison of Amino Acid Accumulation by Pancreas from Various Species

Source of Pancreas	Uptake (μmoles/g tissue water)			
	ACPC	Glycine	AIB	L-Methionine
Mouse	18.2	21.5	18.4	11.3
Rat	15.8	16.7	16.1	10.4
Guinea Pig	3.9	18.8	11.5	2.8
Dog	3.6	10.6	8.8	2.7
Human pancreatic tumor	4.9	4.6	4.2	-

A Ca^{++}-free Krebs-Ringer phosphate medium containing 2 mM radioactive amino acid was used throughout. The incubations were carried out at 37°C for 60 minutes as described in "Materials and Methods".

With dog and human, slices were used, while in the case of mouse, rat and guinea pig small strips of pancreas were employed. The uptake was calculated (as described in "Materials and Methods") on the basis of total water content as a percentage of the wet tissue weight. These values were: mouse 75%, rat 75%, guine pig 78%, dog 78% and human 76%. The values for the human tumor are the results of one experiment carried out in triplicate.

acids were used to compare the accumulation of amino acids by pancreas from various mammalian species. It was evident, that mouse pancreas possessed the greatest capacity for accumulation, although in several instances this was almost equalled by rat pancreas. In contrast to mouse and rat, guinea pig pancreas did not accumulate all the neutral amino acids very well as indicated by distribution ratios (tissue concentration/medium concentration) of 2.0 and 1.4 for ACPC and L-methionine respectively. A similar picture was observed with dog pancreas where glycine and AIB were highly concentrated and low distribution ratios were obtained for the two other amino acids. In the case of the human pancreatic tumor, uptakes of the several amino acids were extremely low compared with the other pancreatic sources. It was not possible to compare this preparation with a normal human pancreas due to the difficulty in obtaining such a control tissue.

In the in vivo situation there is simultaneous entry of several amino acids into the cell and therefore the effects of a number of amino acids on the transport of a model amino acid were examined. For these experiments AIB was used since it undergoes negligible metabolic transformation. The values shown in Table 3 are expressed as a percentage of the uninhibited AIB uptake for mouse, rat and guinea pig pancreas. Several similarities and dissimilarities are evident. In the case of the aromatic amino acids, L-tryptophan and L-phenylalanine, a much greater decrease was seen with rat pancreas than with mouse or guinea pig pancreas, indicating that in the former system there was a greater affinity for the site involved in AIB uptake. With glycine as the inhibitory amino acid, rat

TABLE 3

The Effects of Several Amino Acids on the Uptake of AIB-1-C^{14} by Pancreas from Various Species

Amino Acid Added (5 mM)	Mouse Pancreas		Rat Pancreas		Guinea Pig Pancreas	
	$(A_c - A_f)_{mM}$	% Control	$(A_c - A_f)_{mM}$	% Control	$(A_c - A_f)_{mM}$	% Control
Nil	17.9	100	16.6	100	8.5	100
Glycine	10.5	58	12.0	72	6.1	72
ACPC	8.7	48	10.6	64	2.2	26
L-Phenylalanine	15.5	86	11.9	71	8.3	97
L-Tryptophan	16.9	94	11.3	67	8.8	103
L-Proline	10.8	59	12.0	72	3.8	45

The incubation was carried out at 37°C for 90 minutes as described in Table 2 and "Materials and Methods". The AIB-1-C^{14} was present at an external concentration of 2 mM. The type of tissue preparation and total water content of the tissues were the same as described in Table 2. A_c and A_f are the tissue and extracellular amino acid concentrations respectively.

and guinea pig pancreas were affected to the same extent, while mouse pancreas was slightly more sensitive to inhibition at this concentration. In the presence of ACPC and L-proline the least inhibited of all three appeared to be rat pancreas. Thus there are not only maximal uptake differences, but also several dissimilar patterns of interaction among amino acids, so that a situation shown to exist in mouse pancreas need not necessarily be applicable to pancreas from other mammalian species.

3.3 Comparative Amino Aid Uptake based on Similar Tissue Structure

The pancreas, a racemose type of gland, contains numerous acini, each comprised of cells surrounding a central cavity into which the glandular secretion is received. The duct leading from one follicle joins a similar one from a neighbouring follicle to form a larger channel which unites in turn with others of the same size. Successive unions of this kind result in the formation of a system of ducts with the secretion being discharged to the surface ultimately through a single or a few large ducts. Since the salivary gland not only possesses a similar type of structure, but also synthesizes a number of digestive enzymes, it was of interest to compare these two organs as far as amino acid accumulation was concerned. In this series of experiments (Table 4) the glands of mice and rats were compared. There was an active transport system in the salivary gland (submaxillary) as indicated by the inhibition of the uptake of glycine by AIB and DNP. However, the accumulated uptake levels of glycine and ACPC were very much lower in the salivary gland than in the pancreas of both animals. Furthermore, similar experiments

TABLE 4

Comparison of Amino Acid Uptake by the Pancreas and Salivary Gland of Mouse and Rat

Addition to the External Medium	Amino Acid Uptake (μmoles/g tissue water)			
	Mouse Salivary Gland	Mouse Pancreas	Rat Salivary Gland	Rat Pancreas
ACPC - C^{14}	2.5	18.2	2.9	15.8
Glycine - C^{14}	3.1	21.5	8.0	16.7
Glycine-C^{14} + 6 mM AIB	-	-	5.5	-
Glycine-C^{14} + 0.1 mM DNP	-	-	5.8	-

The incubation was carried out at 37°C for 60 minutes as described in "Materials and Methods". Strips of rat and mouse pancreas, and slices of mouse and rat salivary gland (submaxillary portion) were used. Uptake is expressed as μmoles of amino acid per gram tissue water, calculated on the basis of total water content as a percentage of the wet tissue weight as described in "Materials and Methods". These values are: mouse salivary gland 82%, mouse pancreas 75%, rat salivary gland 83%, and rat pancreas 75%.

(not quoted) with the sublingual gland (a salivary gland) also showed a much lower amino acid uptake than the pancreas.

SUMMARY OF CHAPTER 3

(1) Swiss white mice weighing 20 or 40 g, whether on a regular diet or fasted for 72 hours prior to experimentation, gave almost identical values for the accumulation of ACPC.

(2) Pancreas from newborn mice and adult mice bearing an Ehrlich ascites tumour gave lower uptakes than pancreas from normal adult mice.

(3) Mouse pancreas showed a greater amino acid accumulation than rat, guinea pig, dog or human pancreas.

(4) Comparison of mouse pancreas with pancreas of other mammalian species showed that there were both similarities and dissimilarities in amino acid interaction during transport.

(5) The uptake of amino acids was greater in pancreas than in the salivary gland.

CHAPTER 4

GENERAL ASPECTS OF STEADY-STATE AMINO ACID TRANSPORT IN MOUSE PANCREAS

4.1 The Time Course of Uptake

Since the studies in this and subsequent chapters require the estimation of tissue radioactivity at steady-state levels, a time course of uptake was undertaken for each of the individual amino acids. These results are presented in Fig.1. In the case of L-tryptophan and AIB, the steady-state level was reached in approximately 90 minutes. Although L-lysine was not accumulated to the same elevated levels as the two previous amino acids, no further increase in the tissue concentration was observed after 60 minutes incubation. In contrast, the steady-state level of GABA was attained after 30 minutes with a large portion of the uptake presumably being due to diffusion processes because of the rather limited accumulation. Each time course was carried out as a separate series of experiments and is presented on the same graph simply for comparison purposes.

The time courses of the uptakes of the other amino acids used in these studies have been determined by Bégin and Scholefield (46-48), and the periods of incubation are: glycine, ACPC and L-methionine, 60 minutes; L-proline, 120 minutes.

4.2 Chromatography of the Tissue Extracts

A determination of the amino acid metabolism during the course of the experiment was essential since the tissue concentration of the

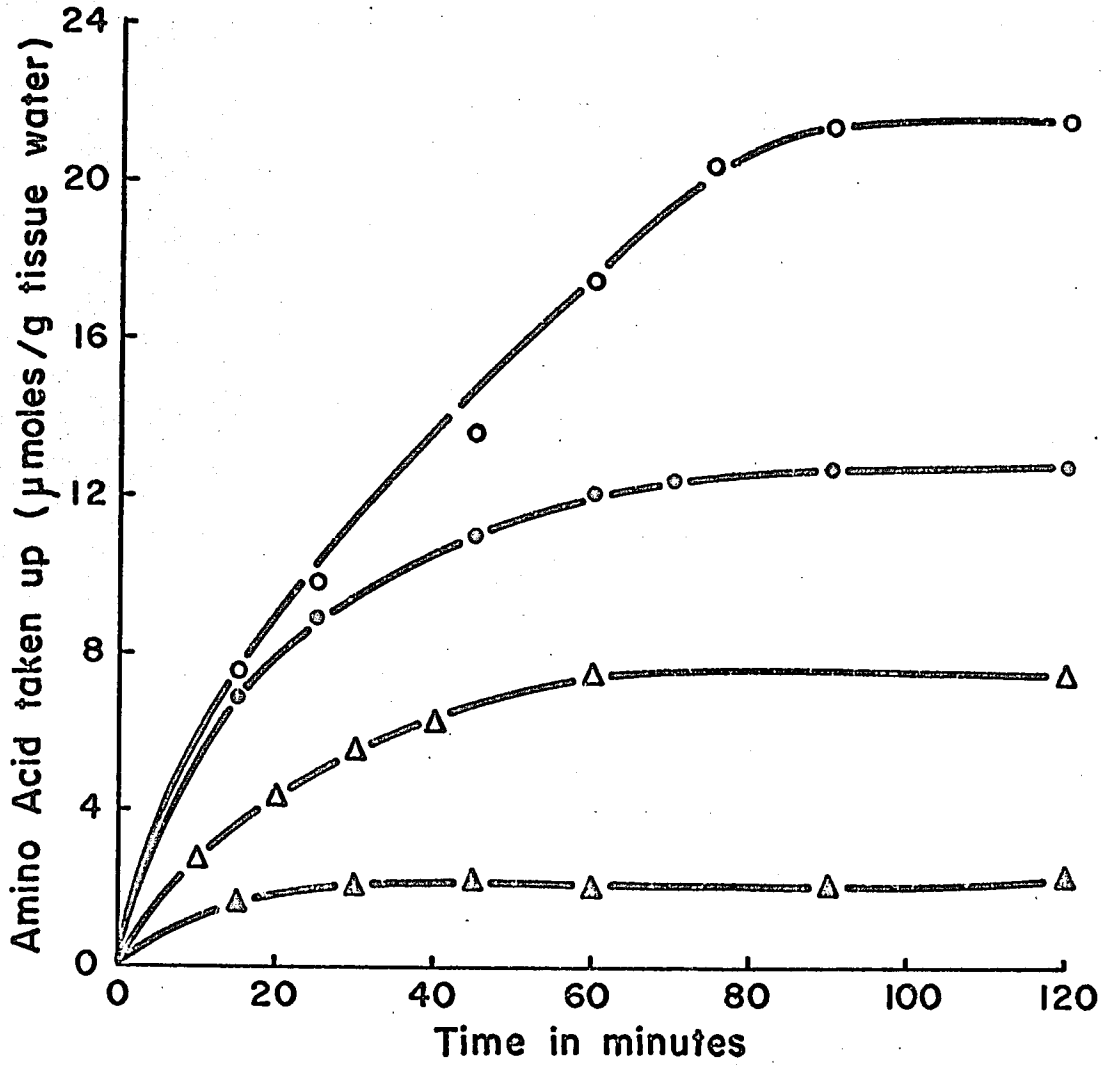

FIG.1: The time course of uptake of amino acids into mouse pancreas: GABA (closed triangles); L-lysine (open triangles); L-tryptophan (closed circles); AIB (open circles).

Incubations were carried out as described in "Materials and Methods". All the amino acids were present in the external medium at a concentration of 2.0 mM.

amino acids was calculated on the basis of tissue radioactivity. In this procedure it was assumed that the amino acid was not metabolized to any significant extent and that the radioactivity in the tissue was therefore a measure of the amount of original compound. Table 5 summarizes the results of paper chromatographic separation of an alcohol extract of mouse pancreas strips following incubation at 37°C with each of the labelled amino acids. The data are expressed as the percentage of the total radioactivity in the extract that was found in the spot corresponding to the respective amino acid. Most of the amino acids were present in excess of 95% of the original starting material, while L-lysine was present intracellularly in a minimum of 90% of its original form. Under these experimental conditions no corrections in the calculations were applied for the metabolism of the amino acids.

4.3 Analysis of the Endogenous Free Amino Acid Content of Mouse Pancreas

The exocrine portion of mouse pancreas is involved in the extensive synthesis of a number of digestive enzymes and so there would exist the need for available pools of amino acids within the tissue. Thus, the penetrating amino acid does not enter a totally amino acid free environment. An analysis of the extent of these pools (Table 6) was carried out by Dr. Charles R. Scriver of The de Belle Laboratory for Biochemical Genetics of The Montreal Children's Hospital. The values for the individual amino acids are expressed as μmoles per wet weight of tissue and therefore represent both intracellular and intercellular amino acid. In the case of amino acids such as hydroxyproline, β-alanine, L-phenylalanine,

TABLE 5

Chromatographic Analysis of the Metabolism of C^{14} - Amino Acids

Amino Acid	Per Cent Radioactivity Unchanged
L-Tryptophan	95
AIB	> 99
GABA	95
L-Lysine	90
Glycine	> 99
L-Methionine	95
L-Proline	95
ACPC	> 99

The pancreas strips were incubated in a medium containing 2 mM of each of the C^{14}-amino acids listed. Chromatography was carried out as described in "Materials and Methods".

TABLE 6

The Endogenous Free Amino Acid Content of Mouse Pancreas

Amino Acid	Tissue Concentration μmoles/g wet weight of tissue
Hydroxyproline	trace
β -Alanine	trace
Phenylalanine	0.014
Isoleucine	0.034
Leucine	0.034
Histidine	0.107
Methionine	0.122
Arginine	0.147
Proline	0.259
Tyrosine	0.283
Ornithine	0.308
Valine	0.335
Serine	0.411
Lysine	0.493
Asparagine, Glutamine	0.609
Alanine	1.585
Threonine	1.966
Glycine	3.093
Taurine	3.185
Glutamic Acid	4.160

Pancreas tissue from four mice was pooled and homogenized in 6 ml of picric acid. The sample was centrifuged, the picric acid removed and a portion of the supernatant processed on an amino acid analyzer.

isoleucine, leucine and histidine, the levels were 0.1 μmoles/g wet weight of tissue or less, while with amino acids such as methionine, proline, lysine and tyrosine, the endogenous level was in the range of 0.1 to 0.5 μmoles/g wet weight of tissue. As shown in the lower portion of Table 6, alanine, threonine, glycine, taurine and glutamic acid were present in relatively high concentrations. Nearly all of the amino acids used in these studies were present at quite low levels within the tissue.

4.4 The Entry of AIB

Previous work by Bégin and Scholefield (48) with mouse pancreas had demonstrated that ACPC, an unnatural amino acid, was transported by a system distinct from that serving for glycine or L-methionine entry. The transport of AIB, another unnatural amino acid and structural analog of glycine, was then studied by employing the ABC test (229, 230) whose criteria are as follows: If two amino acids termed A and B are transported by the same system then (1) they should inhibit each other's uptake and the inhibition should be competitive, (2) since the same site is involved, the K_M value of amino acid A as a substrate should be the same as its K_I value as an inhibitor of B, (3) similarly the K_M value of B as a substrate should be the same as its K_I value as an inhibitor of A, and (4) a third amino acid C should have the same K_I when taken as an inhibitor of A or B.

The K_M values reported were taken directly from Lineweaver and Burk plots. The K_I values were calculated using a point on the line

best fitting competitive inhibition and the equation:

$$\frac{A}{A_c - A_f} = 1 + \frac{K_M}{A_f}\left(1 + \frac{I}{K_I}\right)$$

where A is the maximum concentration gradient, I is the inhibitor concentration, K_M and K_I are Michaelis-Menten type constants, and A_c and A_f are the intracellular and extracellular substrate concentrations respectively.

Results were plotted according to the method of Lineweaver and Burk (231) as illustrated by the inhibition of AIB uptake by glycine (Fig.2). All values represent the mean of at least 12 separate determinations. As can be seen from this figure, the criteria of competitive inhibition is fulfilled. However, enzyme kinetic studies indicate that the analysis of the transport data thus far obtained may be incomplete.

In the true competitive situation, where the inhibitor and substrate combine with the same site, the enzyme may be in combination with either substrate or inhibitor, but not both. It is also possible, however, that the inhibitor, while not combining with the substrate-binding site, may combine with another site sufficiently close to reduce the affinity of the enzyme for the substrate (232). It will thus produce competitive effects, although the enzyme can still be combined with both substrate and inhibitor at the same time. The EIS complex, when formed, is reorientated at the same rate as the ES complex in this type of system. This situation is completely indistinguishable from the true competitive type merely by varying the substrate concentration at fixed

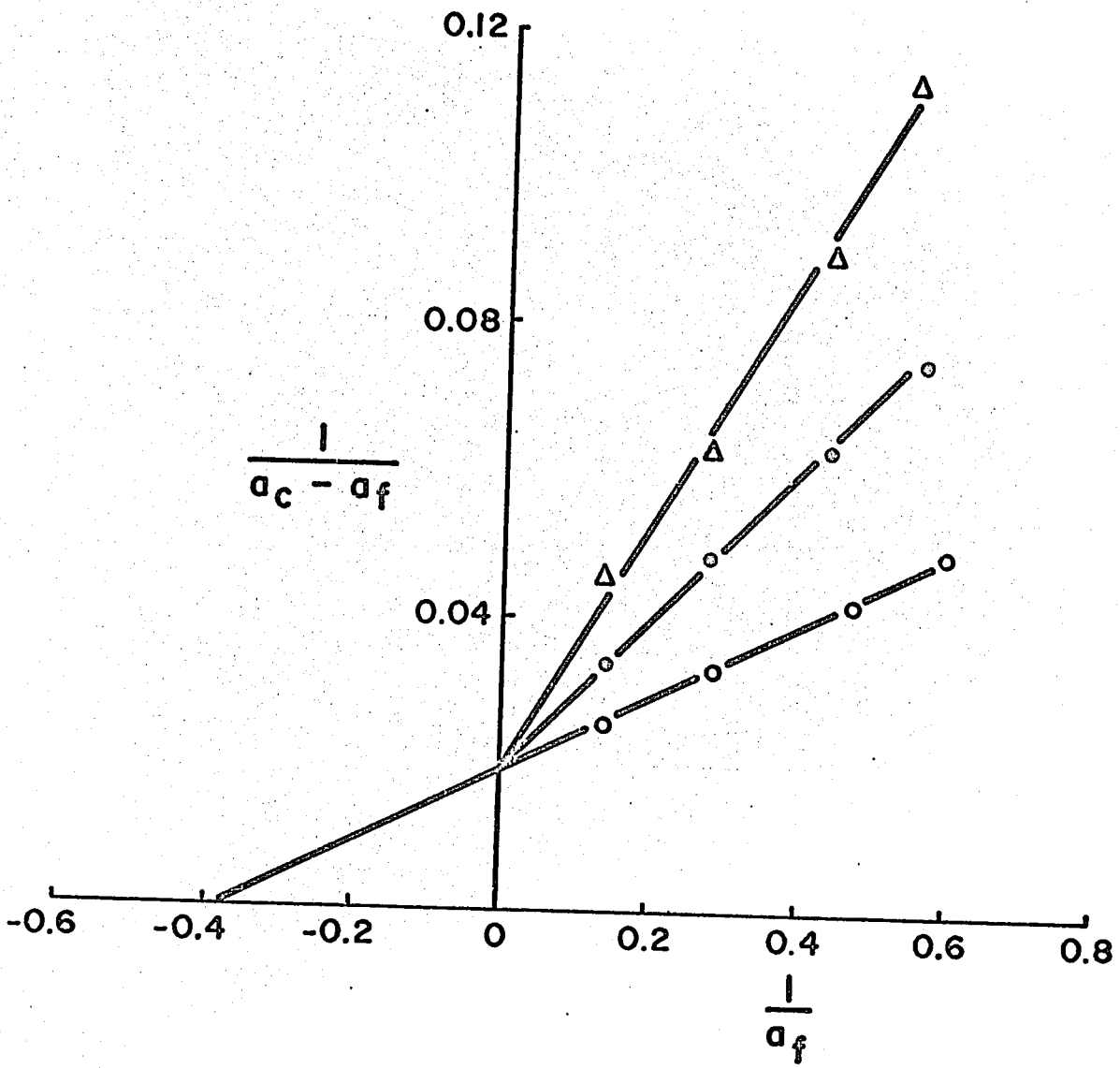

FIG. 2: The effects of substrate concentration and of glycine on the uptake of AIB-1-C^{14}. AIB only (open circles); 4 mM glycine added (closed circles); 8 mM glycine added (open triangles).

The incubation was carried out at 37°C for 90 minutes as described in "Materials and Methods". A_c and A_f are the intracellular and extracellular concentrations of AIB respectively, expressed as millimolarities.

inhibitor concentrations, as in the Lineweaver and Burk graphical analysis shown previously. It may, however, be distinguished by varying the inhibitor concentration at fixed substrate concentration, for the inhibition does not increase indefinitely with increase of inhibitor concentration (as in the true competitive case). but increases to a definite limit when all the enzyme is combined with inhibitor, and can then increase no further. Thus a graphical plot of the reciprocal of the uptake against the inhibitor concentration will not give a straight line, as it does in the true competitive case.

Fig.3 shows such a graphical plot for the interaction between the amino acids AIB and glycine, when used as substrate and inhibitor respectively. The substrate was kept constant at 2 mM and the inhibitory amino acid (glycine) was varied between 0 and 8 mM in the incubation medium. Over this range of inhibitor concentration, the plot was linear. This clearly indicates that, in mouse pancreas, interaction between these two amino acids is primarily due to competition for the substrate site rather than to allosteric interference by inhibitor attachment to another site.

A summary of the K_M and K_I values for glycine - AIB interaction are presented in Table 7. The K_I value for each inhibitory amino acid was determined from a Lineweaver and Burk analysis at two different concentrations of the inhibitor. The K_M and K_I values for AIB of 2.7 and 3.3 respectively agree quite favorably, while the K_M and K_I values for glycine of 4.2 and 3.6 respectively are also in good agreement. The

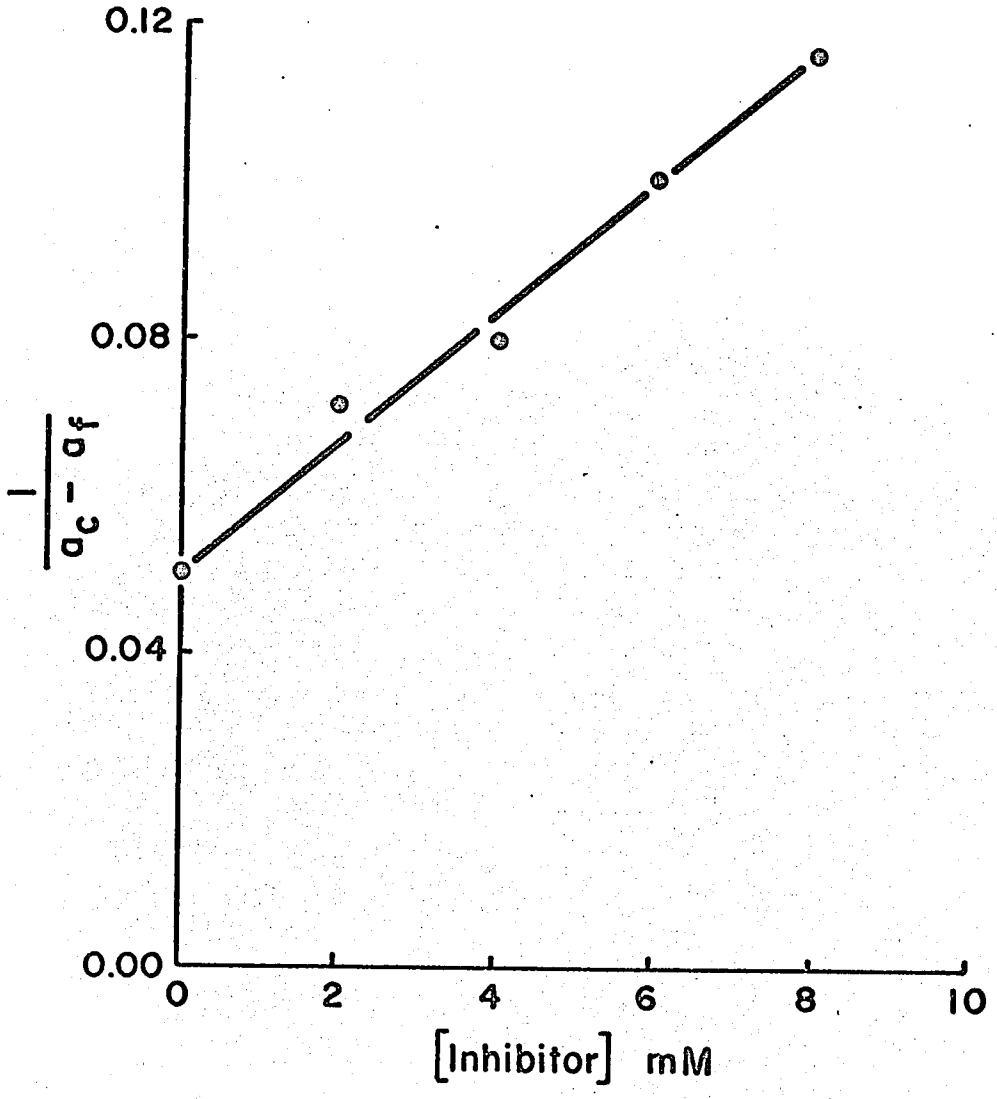

FIG.3: The effects of increasing concentrations of glycine on the uptake of 2 mM AIB-1-C^{14}. The incubation was carried out at 37°C for 90 minutes as described in "Materials and Methods". A_c and A_f are the intracellular and extracellular amino acid concentrations respectively.

TABLE 7

Comparison of K_m and K_I values for AIB - Glycine Interaction in Mouse Pancreas

Inhibitor	Substrate	
	AIB	Glycine
AIB	<u>2.7</u>	3.3
Glycine	3.6	<u>4.2</u>
ACPC	2.4	4.4

The underlined values are the K_m values, i.e. the affinity of amino acids for their own transport sites. The other values are K_I values. All K_m and K_I values were obtained as described in the text.

only discrepancey appears in the inhibitory effect of ACPC on each of the substrate molecules, which in the case of AIB is 2.4 and with glycine 4.4. Thus although there is fairly close agreement among the K_M and I_I values for the interaction of the amino acids, the effect of the third amino acid (ACPC) suggests that AIB entry occurs at a separate site from glycine.

It is possible that AIB may enter via one of the other two systems, namely the one serving for ACPC, MeACPC and L-valine, and the other for L-methionine and L-ethionine. Because the K_I value for ACPC on L-methionine uptake is 6.1 (48), while as an inhibitor of AIB uptake (in these present studies) has a value of 2.4, the L-methionine pathway is definitely excluded. Similarly, since the K_I for glycine on ACPC uptake is 8.4 (48) while the K_I for glycine on AIB uptake is 3.6, the possibility that entry is by means of the ACPC system is also discounted. Therefore, on the basis of the "ABC test" the existance of an additional site must be postulated to account for the entry of AIB into mouse pancreas.

4.5 The Transport of L-Tryptophan

The uptake of L-tryptophan at different concentrations was measured and the results were plotted according to the method of Lineweaver and Burk (231) as shown in Fig.4. The values, when plotted by this method, did not give a straight line over the whole range of concentrations. It was possible to draw two separate straight lines through a restricted number of points, with approximately 1.0 mM substrate concentration being

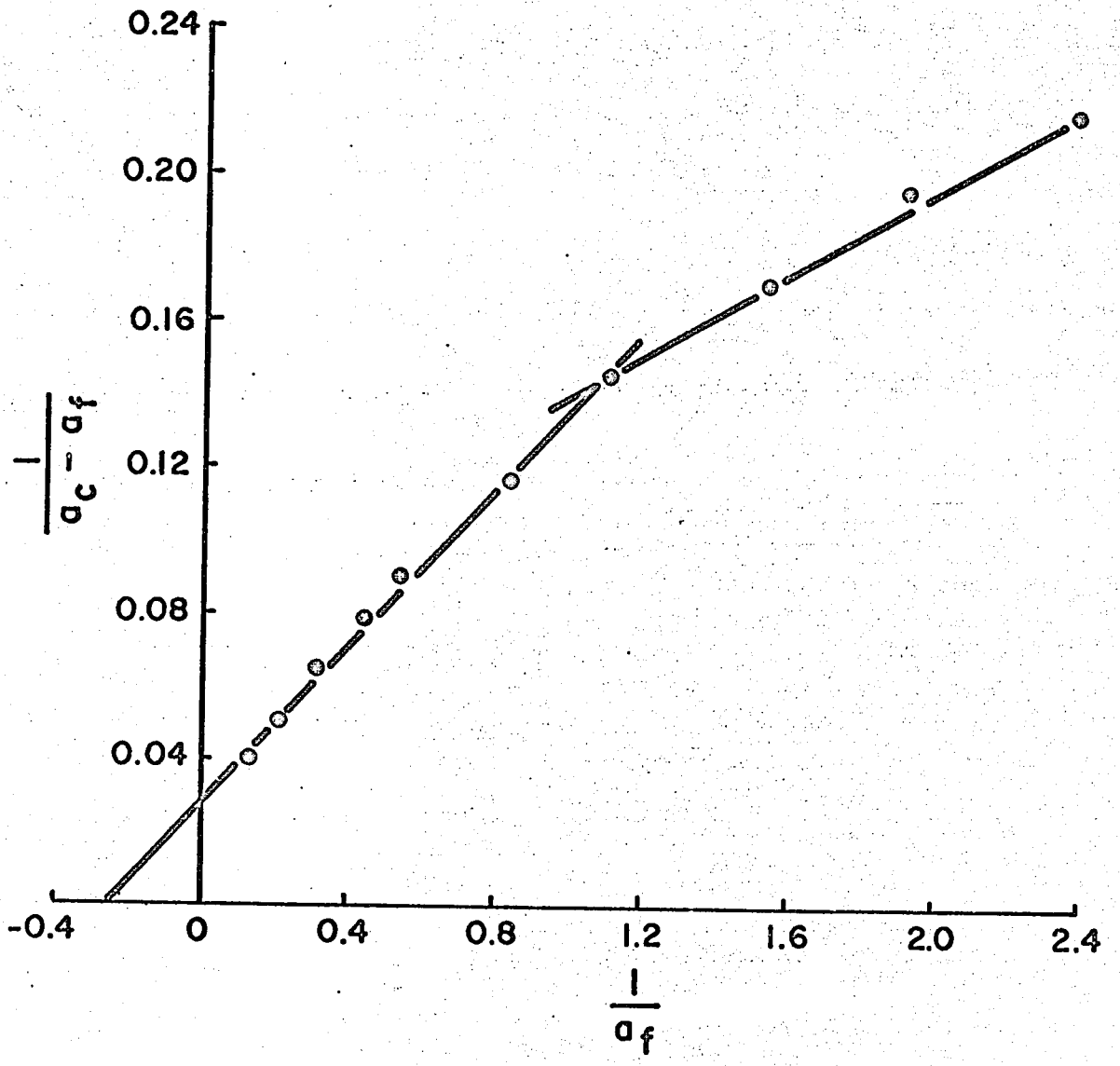

FIG.4: The effects of substrate concentration on the uptake of L-tryptophan-3-C^{14}. A_c and A_f are the intracellular and extracellular amino acid concentrations expressed as millimolarities. The incubation was carried out at 37°C as described in "Materials and Methods".

the region of intersection. This type of result suggests that L-Tryptophan may have at least two modes of entry into mouse pancreas i.e. the first operating at low substrate concentrations with a K_M of 0.6 mM and a maximum concentration gradient of 11 mM and the second at higher concentrations with a K_M of 3.8 mM and a maximum concentration gradient of 33 mM. These routes are referred to as tryptophan I and tryptophan II respectively. The kinetic constants are only approximate values since the two systems overlap each other to some extent.

Although a non-linear Lineweaver and Burk plot was detected for L-tryptophan and not for any of the amino acids previously studied (46, 48), it should be noted that earlier studies had not extended the concentration range below 2 mM. Since the additional pathway for L-tryptophan uptake was detected at substrate concentrations below this value, it was advisable to re-examine a Lineweaver and Burk plot of some of the other amino acids over a wider range of concentrations (not quoted). The amino acids, ACPC and glycine, representatives of two different pathways (48), were selected. In both cases the concentration range was extended as low as 0.5 mM and in no instance was there any deviation resembling that found with L-tryptophan. The K_M and maximum concentration gradient values were the same as those reported by Bégin and Scholefield (48).

4.6 The Transport of L-Lysine

A Lineweaver and Burk analysis of L-lysine uptake (Fig.5) indicated a basic difference from the amino acids studied previously.

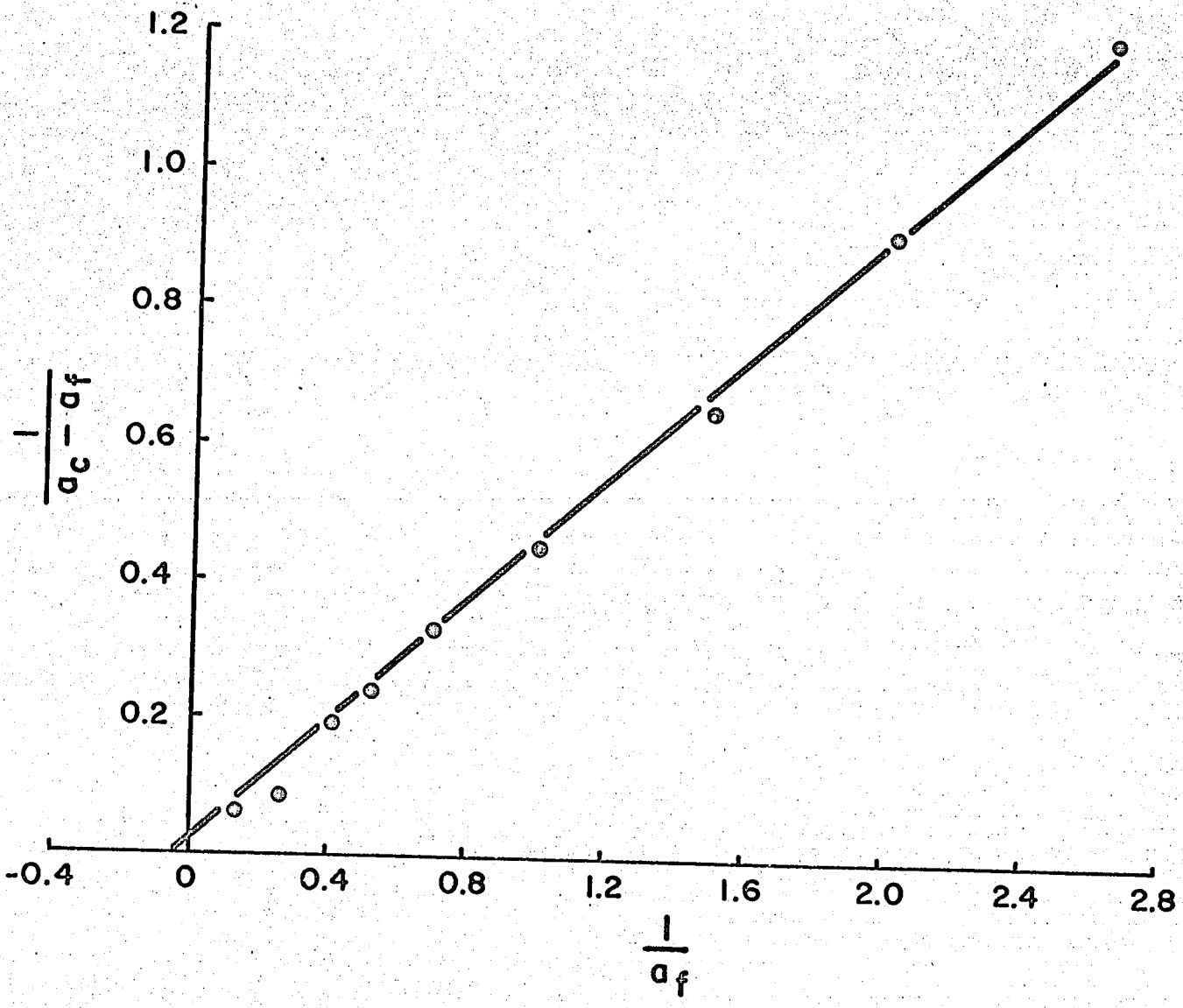

FIG. 5: The effects of substrate concentration on the uptake of L-lysine-U-C^{14}. A_c and A_f are the intracellular and extracellular amino acid concentrations expressed as millimolarities. The incubation was carried out at 37°C for 60 minutes as described in "Materials and Methods".

With L-lysine, the plot was linear over the whole range of concentration, although the affinity constant (K_M) was approximately 25 mM. This is a much higher value than any other previously reported in mouse pancreas (46-48) and would indicate a quite low affinity of lysine for its own transport system. This is in contrast to a K_M value of 2.0 mM for L-lysine uptake in kidney slices (233).

4.7 Amino Acid Interaction During the Transport Process

There is a great deal of interaction between amino acids during the transport process as illustrated by glycine and AIB in Section 4.4 and the results in Table 8. Here the effects of a limited number of possible inhibitory amino acids were studied using a three-fold excess of inhibitor over substrate. As can be seen, the best inhibitors of L-lysine transport were L-ethionine and L-phenylalanine, while GABA, at the same concentration, had no effect. This is in contrast to GABA being a significant inhibitor of the uptake of L-methionine, glycine, AIB and ACPC. Although the tissue steady-state level of GABA was extremely low, carrier-mediated entry was still involved as shown by the inhibitory effects of several amino acids. β-Alanine, as an inhibitor, had approximately the same effect on all of the amino acids, including L-lysine. The fifth column of the table shows the effect of six amino acids on L-tryptophan uptake. In the presence of glycine, taurine and GABA, slightly stimulated steady-state levels were obtained. L-Proline as an inhibitory amino acid was less effective in the case of L-tryptophan accumulation than with L-methionine, glycine, AIB or ACPC accumulation. In addition, the uptakes

TABLE 8

The Inhibition of Pancreatic Amino Acid Accumulation by Several Neutral Amino Acids

Inhibitor	Substrate (uptake*)						
	L-Lysine	GABA	L-Methionine	L-Tryptophan	Glycine	AIB	ACPC
Nil	7.00	2.02	10.57	12.64	24.52	19.96	15.13
Glycine	5.65(19)	1.50(25)	-	15.22(+20)	-	11.14(44)	10.36(32)
L-Valine	5.73(18)	1.57(22)	8.58(19)	-	-	10.30(48)	-
L-Ethionine	3.64(49)	1.75(13)	6.30(40)	-	-	5.91(70)	6.93(54)
Taurine	-	-	10.34(2)	14.59(+15)	21.96(10)	18.71(6)	13.33(12)
GABA	7.08(0)	-	8.77(17)	13.73(+9)	19.70(20)	14.69(26)	12.77(15)
AIB	5.73(18)	-	8.07(23)	11.74(9)	12.64(48)	-	10.42(31)
β-Alanine	6.31(9)	1.81(10)	11.62(+10)	11.26(11)	21.48(12)	17.92(10)	13.59(10)
L-Proline	-	1.66(17)	7.39(30)	11.37(10)	15.38(36)	13.09(34)	11.85(22)
L-Threonine	-	-	8.88(16)	-	15.30(37)	11.62(42)	8.91(41)
L-Leucine	-	-	7.05(33)	-	15.28(37)	-	7.87(48)
L-Phenylalanine	4.70(33)	1.60(21)	6.87(35)	-	-	13.49(32)	-
L-Serine	-	-	-	-	-	8.65(57)	9.88(35)
ACPC	6.43(8)	2.02(0)	-	-	-	10.43(48)	-

* Uptake is expressed as μmoles/g tissue water

The incubations were carried out at 37°C as described in Section 4.1 and "Materials and Methods". Labelled amino acids were present at a concentration of 2 mM and the inhibitory amino acids at a concentration of 6 mM.

All values are the mean of at least six determinations. The numbers in parentheses represent the percentage change (inhibition or stimulation +) from the control amino acid uptake.

of all the neutral amino acids, with the exception of L-tryptophan, were poorly inhibited by the presence of taurine.

Christensen (149) found that in the Ehrlich ascites cell "there was major suppression of lysine transport by a sufficient level of any neutral amino acid,whereas an excess of lysine eliminated only a clearly defined portion of the neutral amino acid uptake." The subsequent tables show the results of experiments using an excess of inhibitor amino acid i.e. increasing concentrations up to a 60-fold excess of inhibitor over substrate. In all these studies with mouse pancreas the external sodium chloride concentration, normally 145 mM, was kept constant at 105 mM. The inhibitory amino acid replaced a portion of the added choline chloride in order to maintain an iso- osmotic medium.

(A) <u>The Effects of Excess Neutral Amino Acids on the Uptake of Another Neutral Amino Acid.</u>

Table 9 shows the effect of increasing concentrations of the neutral amino acids glycine, AIB and L-methionine on the uptake of ACPC. Although each of the inhibitory amino acids is transported at a separate site from that serving for ACPC entry (48), there was major suppression of the uptake of ACPC by a sufficient concentration of all the neutral amino acids. The decrease was expressed as percentage inhibition of the control amino acid accumulation and was calculated on the basis of the concentrative accumulation i.e. accumulation above the medium concentration. At an inhibitor concentration of 60 mM this reduction amounted to

TABLE 9

The Effects of Increasing Concentrations of Neutral Amino Acids on the Uptake of ACPC

Concentration of Inhibitor (mM)	Uptake of ACPC (μmoles/g tissue water) when the inhibitor is		
	Glycine	AIB	L-Methionine
0	7.37	7.35	7.36
4	6.60	6.39	4.10
10	4.13	3.98	2.83
25	2.54	2.67	1.99
40	2.17	2.12	1.87
60	1.93	1.72	1.81

The incubation was carried out at 37°C for 60 minutes as described in "Materials and Methods". ACPC-C^{14} was present at an external concentration of 1 mM. The NaCl concentration was kept constant at 105 mM and the inhibitory amino acid replaced a portion of the added choline chloride.

approximately 85% with glycine, 89% with AIB and 87% with L-methionine.

(B) The Effects of Excess Neutral Amino Acids on the Uptake of a Basic Amino Acid.

The effect of increasing concentrations of neutral amino acids on the uptake of L-lysine is shown in Table 10. At the maximum 60 mM inhibitor concentration the reduction amounted to approximately 52% with glycine, 40% with ACPC and 60% with L-methionine. These values are lower than the excess neutral on neutral effect seen in the previous section.

(C) The Effects of Excess Basic Amino Acid on the Uptake of Several Neutral Amino Acids.

The effect of excess L-lysine on ACPC, glycine and L-methionine uptake is shown in Table 11. Although a 60 mM external concentration of L-lysine inhibited the uptake of L-methionine and glycine 13% and 25% respectively, the accumulation of ACPC was reduced approximately 57%, indicating that a sufficient concentration of the basic amino acid could eliminate either a small or large portion of neutral amino acid uptake depending upon the substrate under consideration. This is therefore not in agreement with the findings of Christensen and his co-workers as reported for Ehrlich ascites cells (149).

As noted previously in Table 9, inhibition of neutral amino acid uptake by other neutral amino acids was quite extensive. The possibility of this inhibition still occurring in the presence of excess basic amino acid is examined in Table 12. As can be seen, the uptakes

TABLE 10

The Effects of Increasing Concentrations of Neutral Amino Acids on the Uptake of L-Lysine

Concentration of Inhibitor (mM)	Uptake of L-Lysine (μmoles/g tissue water) when the inhibitor is		
	Glycine	ACPC	L-Methionine
0	3.04	3.04	3.15
4	2.67	2.94	2.28
10	2.39	2.56	1.91
25	2.11	-	-
40	-	2.39	1.93
60	1.98	2.21	1.86

The incubation was carried out at 37°C for 60 minutes as described in "Materials and Methods". L-Lysine-C^{14} was present at an external concentration of 1 mM. The Na^+ concentration was maintained as described in Table 9.

TABLE 11

The Effects of Increasing Concentrations of L-Lysine on the Uptake of Several Neutral Amino Acids

Concentration of L-Lysine (mM)	Uptake (μmoles/g tissue water)		
	ACPC	Glycine	L-Methionine
0	9.06	9.66	5.93
4	9.09	8.55	-
10	7.84	8.25	5.89
25	6.48	-	5.82
40	5.54	8.73	5.86
60	4.47	7.47	5.31

The incubation was carried out at 37°C for 60 minutes as described in "Materials and Methods". Each amino acid was present at an external concentration of 1.0 mM. The Na^+ concentration was maintained as described in Table 9.

TABLE 12

The Effects of a Mixture of L-Lysine and Neutral Amino Acids on the Uptakes of ACPC and Glycine

Amino Acids Added	Uptake (μmoles/g tissue water)	
	ACPC	Glycine
60 mM L-Lysine	3.92, 3.75	7.34, 7.42
60 mM L-Lysine + 20 mM Glycine	2.87, 3.05	-
60 mM L-Lysine + 20 mM L-Methionine	1.97, 2.22	-
60 mM L-Lysine + 20 mM ACPC	-	4.81, 5.41
60 mM L-Lysine + 20 mM AIB	-	4.57, 4.27

Incubation conditions were as described in Table 11. Each set of numbers are the duplicate values obtained in a typical experiment.

of ACPC and glycine in the presence of 60 mM L-lysine were inhibited still further by the addition of other neutral amino acids at an external concentration of 20 mM. The two values for each result show the duplicate determinations in a typical experiment.

(D) The Effects of Excess Taurine and GABA on Neutral and Basic Amino Acid Uptake.

The studies involving an excess of inhibitory amino acid were extended to include the effects of taurine, a sulfonated amino acid, and GABA, an amino acid which is accumulated to a very slight extent (Table 13). Taurine at a 60 mM external concentration caused reductions in ACPC and glycine uptake of 32% and 60% respectively whereas no significant reduction in L-lysine accumulation could be found. With GABA as the inhibitory amino acid, the maximum reductions at a 60 mM concentration were approximately 52% in the case of glycine and L-methionine uptake. When L-lysine was the substrate, there was no decrease in the measurable accumulation. As noted previously, GABA, although being an excellent competitive inhibitor of glycine and L-methionine, is itself poorly transported. The results of Table 13 also indicated that the steady-state accumulation of L-lysine was slightly enhanced by the presence of GABA in the external medium. These observations point out that L-lysine uptake, on the basis of comparative amino acid interaction during transport, behaves differently from that of ACPC, glycine or L-methionine.

TABLE 13

The Effects of Excess Taurine and GABA on the Uptake of Several Amino Acids

Concentration of Inhibitor (mM)	Taurine Inhibition of the Uptake of			GABA Inhibition of the Uptake of		
	ACPC	Glycine	L-Lysine	Glycine	L-methionine	L-Lysine
0	8.57*	11.60	2.86	12.01	5.92	3.31
4	-	9.37	2.66	8.95	5.68	3.98
10	8.96	7.37	2.93	-	5.45	4.00
25	8.70	6.96	3.07	8.04	4.69	4.09
40	6.07	6.31	-	6.35	3.20	-
60	6.22	5.27	2.73	6.31	3.36	4.40

*Uptake is expressed as μmoles/g tissue water.

Incubation conditions were as described in Table 11.

4.8 The Effects of DNP and pH Change on the Transport of Several Amino Acids

In mouse pancreas, grouping of amino acids into different systems has been solely on the basis of kinetic data obtained from an analysis of amino acid interaction during transport. As this phenomenon is carrier-mediated coupled to a source of energy, the effects of DNP and pH were used as additional criteria to further distinguish between the various amino acid transport systems. Table 14 shows the effects of 0.1 mM DNP on the uptake of neutral and basic amino acids all at an external concentration of 1.0 mM. Although L-proline appears to be affected to the greatest extent (67% decrease), the other amino acids do not show any significant differences among themselves with respect to the effect of DNP. On this basis it would be impossible to distinguish between the neutral amino acids, or to differentiate between basic and neutral amino acids since the energy requirement appears to be quite similar for all the substrates.

The effect of pH as a second criterion is shown in Table 15. All the amino acids were studied at an external concentration of 1.0 mM over the pH range 4.8 to 8.1. The uptakes of glycine, ACPC and GABA appeared to be quite insensitive to a change in pH, while in the case of AIB and L-methionine entry, a decrease in pH to 4.8 resulted in uptake reductions of only 11% and 9% respectively (based on pH 7.4 as the control value). However, lowering the pH from 7.4 to 4.8 caused approximately a 38% decrease in the uptake of L-lysine. Furthermore, the ac-

TABLE 14

The Effects of 0.1 mM DNP on Neutral and Basic Amino Acid Uptake

Amino Acid	Control $(A_c - A_f)$ mM	+ DNP $(A_c - A_f)$ mM	% Inhibition
L-Proline	7.62	2.48	67
L-Tryptophan	6.23	2.46	60
AIB	8.11	3.42	58
L-Methionine	4.57	2.10	54
Glycine	11.09	4.70	57
ACPC	8.14	3.59	56
L-Lysine	2.68	1.27	52

The incubation was carried out at 37°C as described in Section 4.1 and "Materials and Methods". A_c and A_f represent the concentrations of amino acid in the tissue and the external medium respectively. Each amino acid was used at an external concentration of 1.0 mM.

TABLE 15

The Effects of pH on the Uptake of Several Amino Acids

Amino Acid	Amino Acid Uptake (μmoles/g tissue water)				
	pH 4.8	pH 5.4	pH 6.2	pH 7.4	pH 8.1
AIB	8.67	-	9.36	9.70	9.02
GABA	0.99	-	0.98	1.05	1.03
ACPC	8.49	-	8.17	8.06	-
L-Methionine	6.17	6.20	6.44	6.78	-
Glycine	12.58	12.90	-	12.80	-
L-Lysine	2.44	2.59	2.70	3.95	4.40

Incubation in sodium phosphate buffers of different pH values was carried out as described in Section 4.1 and "Materials and Methods". All radioactive amino acids were present at an external concentration of 1.0 mM.

cumlation of L-lysine at pH 8.1 was significantly higher than at pH 7.4. Based on the effect of pH, the uptake of L-lysine could easily be differentiated from that of the neutral amino acids. The relative insensitivity of the uptake of the neutral amino acids to pH change is in contrast to the Ehrlich cell where the short-chain neutral amino acids are much more sensitive to pH than the long-chain amino acids (67, 74). In rat kidney cortex slices the uptakes of both glycine and L-lysine are greatly affected by pH (118).

SUMMARY OF CHAPTER 4

(1) The steady-state uptake level was attained after 30 minutes with GABA, 60 minutes with L-lysine and 90 minutes in the case of L-tryptophan and AIB.

(2) The steady-state accumulation levels were: AIB > L-tryptophan > L-lysine > GABA.

(3) The amino acids underwent relatively little transformation to other metabolic products during the course of the incubation.

(4) Analysis of the endogenous free amino acid pools indicated that most of the amino acids were present in low concentrations.

(5) Interaction between glycine and AIB during transport was primarily due to competition for the substrate site rather than to allosteric interference by inhibitor attachment to another site, i.e., no evidence of "partial" competition.

(6) On the basis of the ABC test, the existance of an additional site was postulated to account for the entry of AIB into mouse pancreas.

(7) The non-linear Lineweaver and Burk plot obtained with L-tryptophan suggested that there were at least two modes of entry into mouse pancreas - the first operating at low substrate concentration with a K_M of 0.6 mM and the second at higher concentrations with a K_M of 3.8 mM.

(8) L-Lysine, which gave a linear Lineweaver and Burk plot, had a quite low affinity for its own transport system.

(9) Although the steady-state tissue level of GABA was extremely low, mediated entry was still involved. GABA inhibited the uptake of the neutral amino acids L-methionine, glycine, AIB and ACPC.

(10) Glycine, taurine, and GABA caused slightly stimulated steady-state uptake levels of L-tryptophan.

(11) There was major suppression of neutral amino acid uptake by a sufficient level of other neutral amino acids.

(12) There was a large reduction in basic amino acid uptake by a sufficient level of several neutral amino acids, although this was less than the excess neutral on neutral effect.

(13) A sufficient concentration of a basic amino acid could eliminate either a small or large portion of neutral amino acid uptake depending upon the substrate under consideration.

(14) Taurine at a concentration of 60 mM caused reductions in the uptakes of ACPC and glycine, but not of L-lysine. GABA at a concentration of 60 mM inhibited glycine and L-methionine uptake, but not that of L-lysine.

(15) DNP at a concentration of 0.1 mM caused approximately the same percentage decrease in the uptake of L-lysine and several neutral amino acids.

(16) The uptake of L-lysine was significantly affected by pH, while neutral amino acid uptake was relatively insensitive to pH.

CHAPTER 5

ASPECTS OF THE RELATIONSHIP BETWEEN IONS AND AMINO ACID TRANSPORT IN MOUSE PANCREAS

The close association between electrolyte and non-electrolyte movement in the pancreas has recently been reported by Milner and Hales (208) who found an increased insulin secretion from the β-cells of the pancreas when the normal functioning of the Na^+-K^+ pump was interrupted. This chapter will deal with the participation of Na^+ and K^+ in the active transport of amino acids, a group of compounds which are extensively accumulated by mouse pancreas.

5.1 The Effects of Na^+ and K^+ on Amino Acid Accumulation

Many transport systems have been shown to be dependent upon the concentration of both Na^+ and K^+ in the external medium. Using the model amino acid ACPC, the effects of a wide range of ion concentrations were examined as shown in Fig.6. The concentration of K^+ required for optimum amino acid uptake was approximately the amount in the Krebs-Ringer solution i.e. 7.2 mM. A plot of the uptake of amino acid against the Na^+ concentration also gave a saturation type of curve with an optimum at approximately 145 mM Na^+, while an increase beyond this concentration did not have any inhibitory effect on the transport process. With this particular amino acid, incubation in Na^+ or K^+-free media did not completely eliminate accumulation against a concentration gradient. Thus

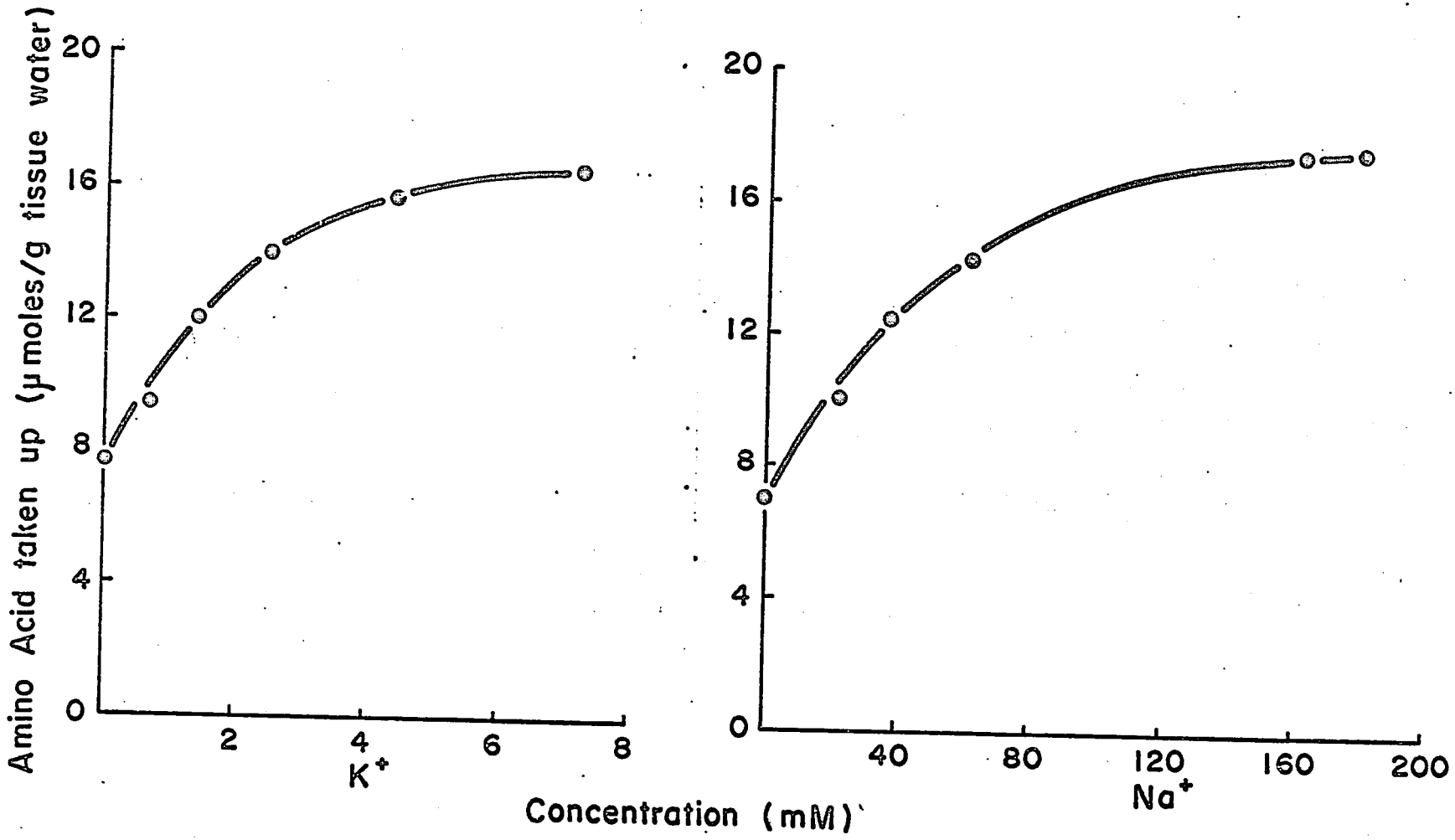

FIG.6: The effects of changes in (a) the external K^+ concentration, and (b) the external Na^+ concentration on the uptake of ACPC-C^{14}. The incubation was carried out at 37°C for 60 minutes with 2 mM ACPC-C^{14} as described in "Materials and Methods". NaCl and choline chloride were used to replace the decreased KCl and NaCl concentrations respectively. For the 0 mM Na^+ concentration, the medium was buffered with tris chloride pH 7.4 instead of sodium phosphate. The Na^+ concentration represents Na^+ from both the Krebs-Ringer solution and the sodium phosphate buffer.

the cation dependent portion of ACPC transport requires the presence of both Na^+ and K^+, since either cation alone is ineffective. Moreover, the activating effect of K^+ outside the membrane is higher than that of Na^+, since the concentration required for half-maximal activity of the cation dependent portion of the uptake was approximately 1.7 mM, whereas the corresponding Na^+ concentration was approximately 38 mM.

5.2 The Effects of Substitution of Other Monovalent Cations

These studies were extended to determine whether other monovalent cations could replace Na^+ or K^+ and still retain the ability to produce high amino acid concentration gradients. Table 16 shows the effects of several ions on the uptake of ACPC. In the case of substitution for the 145 mM NaCl of the external medium, choline chloride and NH_4Cl were more effective than LiCl, RbCl or KCl. Although concentration gradients were maintained in all cases, none of the levels were comparable to those obtained in the presence of NaCl. Substitution for K^+ by other ions, except Rb^+, resulted in a decreased steady-state amino acid accumulation. It appears that Rb^+ can function as a suitable replacement for K^+ under the present experimental conditions. It should also be noted that LiCl and NH_4Cl, when used as substitutes for either Na^+ or K^+, gave almost identical uptake values in both situations. This is in contrast to choline chloride which maintained a higher amino acid concentration gradient when used as a replacement for 145 mM NaCl than for 7.2 mM K^+.

5.3 The Mechanism of Action of Ions on Amino Acid Accumulation

Crane (190) with sugars in intestine, Kipnis and Parrish (205)

TABLE 16

The Effects of Substitution of Other Monovalent Cations for Na^+ and K^+ on the Uptake of ACPC-C^{14}

Substitution Compounds in the Incubation Medium	Uptake of ACPC when Compounds are used to replace	
	145 mM Na^+	7.2 mM K^+
Nil	8.82	8.82
LiCl	3.23	3.45
RbCl	3.57	8.94
NH_4Cl	5.04	4.90
KCl	3.88	-
Choline Chloride	4.91	3.11

The incubation was carried out at 37°C for 60 minutes at an external ACPC concentration of 1.0 mM as described in "Materials and Methods". The medium was buffered with 10 mM sodium phosphate. Substitution cations were used at a concentration of 145 mM replacing the NaCl of the Krebs-Ringer solution. The 7.2 mM K^+ concentration of the Krebs-Ringer solution was replaced by an equimolar concentration of the substitution compounds.

with AIB in lymph nodes and Vidaver (193) with glycine uptake in pigeon red blood cells have shown that an alteration of the external Na^+ concentration results in a change in the apparent affinity constant but not in the maximum velocity of uptake. This is in contrast to the reports of Christensen and his collaborators, who found a change in both the maximum velocity of uptake and the K_M value in such amino acid transport systems as Ehrlich ascites cells, pigeon erythrocytes and rabbit reticulocytes (196). A study was undertaken with mouse pancreas to examine the effects of variations in the external Na^+ and K^+ concentrations on the uptake of AIB, a non-metabolized amino acid.

Fig.7 shows the effects of two different concentrations of Na^+ plotted according to the method of Lineweaver and Burk (231). Each gave rise to an unaltered maximum concentration gradient and an increase in the apparent affinity constant from 2.6 mM to 6.7 mM at the lowest Na^+ concentration. These observations are in direct contrast to the work of Christensen and his collaborators. However, it was possible that the results obtained with this particular amino acid were not representative of amino acid-ion interaction in the pancreas. A second amino acid, ACPC, was then studied since it entered the tissue by a pathway other than that serving for AIB entry (see Chapter 4). The effects of four different concentrations of Na^+ on ACPC uptake is shown in Fig.8a. The unaltered maximum concentration gradient and the decreased affinity constant are in agreement with that obtained for AIB. Furthermore, other experiments (not quoted) carried out in the complete absence of external Na^+ similarly showed only a change in the apparent affinity constant. Changes in the

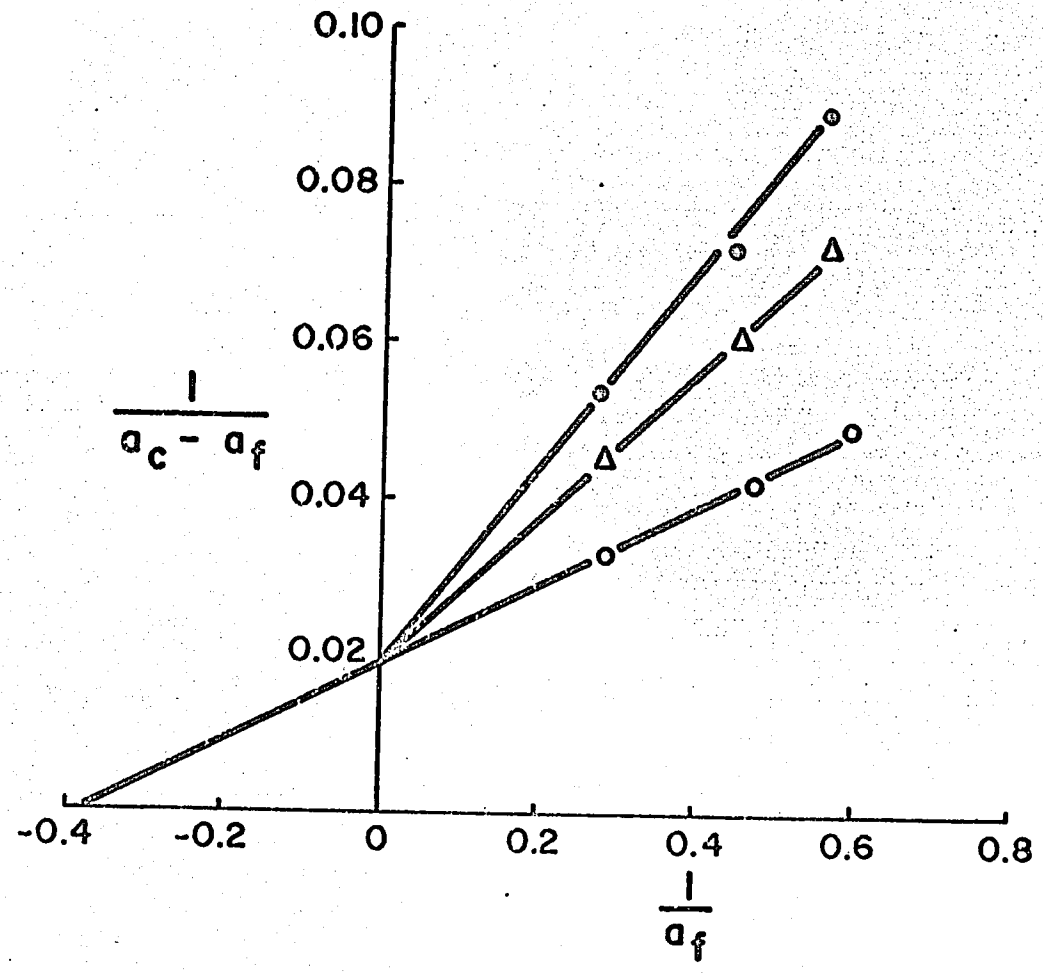

FIG. 7: The effects of a decreased external Na^+ concentration on the uptake of AIB-1-C^{14}. Standard medium (open circles); 67 mM Na^+ (open triangles); 37 mM Na^+ (closed circles). Removed NaCl was replaced by an equimolar concentration of choline chloride.
The incubation was carried out at 37°C for 90 minutes as described in "Materials and Methods". A_c and A_f are the intracellular and extracellular amino acid concentrations respectively, expressed as millimolarities.

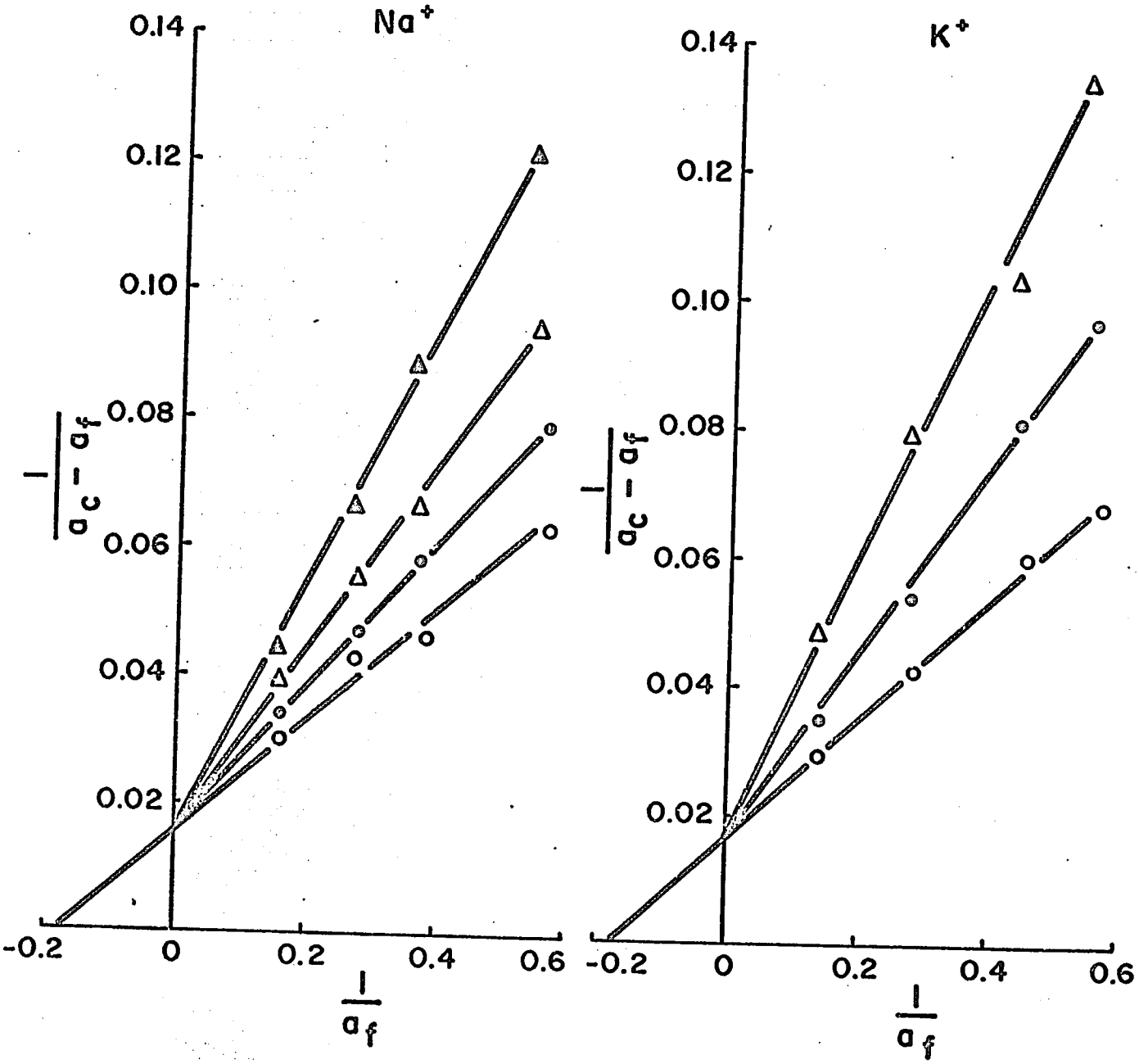

FIG.8: The effects of a decreased external (a) Na^+ concentration, and (b) K^+ concentration on the uptake of ACPC-C^{14}. (a) Standard medium (open circles); 62 mM Na^+ (closed circles); 37 mM Na^+ (open triangles); 22 mM Na^+ (closed triangles). Removed NaCl was replaced by choline chloride. (b) Standard medium (open circles); 1.4 mM K^+ (closed circles); 0.7 mM K^+ (open triangles). Removed K^+ was replaced by Na^+. The incubation was carried out at 37°C for 60 minutes as described in "Materials and Methods". A_c and A_f are the intracellular and extracellular amino acid concentrations respectively, expressed as millimolarities.

external K^+ concentration resembled the Na^+ effect insofar as the results, when plotted according to the method of Lineweaver and Burk (231), gave an unaltered maximum concentration gradient and an increased K_M value (Fig. 8b).

5.4 The Number of Na^+ as Co-factor in the Transport Process

As seen in the previous section, Na^+ plays a definite role in the entry of amino acids into the cell. However, the precise number of Na^+ involved in this process has varied from system to system. Vidaver (193), studying glycine uptake into pigeon red cells, found that 2 Na^+ function as co-factor, while Wheeler and Christensen (197) have calculated that 1 Na^+ is required for alanine uptake and 2 Na^+ for glycine uptake in rabbit red cells. In addition, Cotlier and Beaty (206) have shown a 1 Na^+ requirement for AIB uptake in the rabbit eye lens. A similar study was undertaken with mouse pancreas. Fig.9 shows a double reciprocal plot of the uptake of ACPC against the external Na^+ concentration. The linearity of the plot indicates a 1:1 relationship between Na^+ ions and ACPC molecules transported into mouse pancreas.

5.5 The Comparative Effects of a Decreased Na^+ and K^+ Concentration on Amino Acid Accumulation

With mouse pancreas, grouping of particular amino acids as to entry by one system or another has been strictly on a kinetic basis. Studies reported in Chapter 4 on the effects of DNP and pH as alternate criteria, failed to indicate any additional differences between the up-

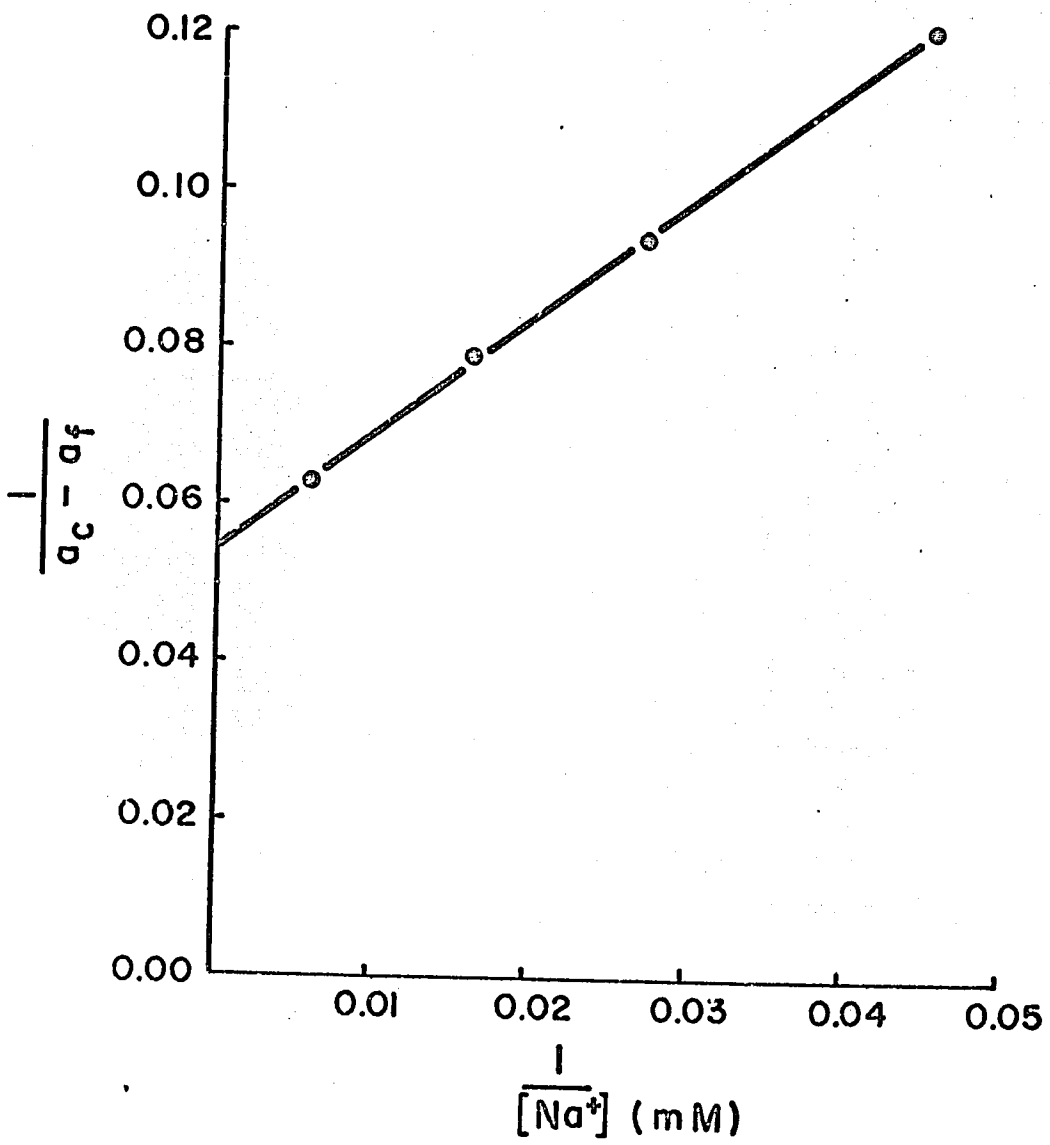

FIG.9: A double-reciprocal plot of ACPC uptake against the initial Na^+ concentration of the medium. The incubation was carried out at 37°C for 60 minutes in the presence of 2 mM ACPC-C^{14} as described in "Materials and Methods". A_c and A_f are the intracellular and extracellular amino acid concentrations respectively. An isoosmotic medium was maintained by choline chloride replacement of the decreased sodium chloride content.

takes of the various neutral amino acids. Table 17A shows the comparative effects of decreased Na^+ and K^+ levels on amino acid uptake. The four neutral amino acids were selected because each, on the basis of the ABC test, was shown to enter via a separate transport site (46). Decreasing the concentration of K^+ to 1.5 mM affected the accumulation of all the amino acids to about the same extent. In the case of Na^+ reduction to 42 mM, AIB uptake was affected to the greatest extent, while ACPC, glycine and L-methionine showed similar reductions in their steady-state levels. However, the actual differences between AIB and the other amino acids were quite small, making it extremely difficult to differentiate between the various transport sites for neutral amino acids on the basis of decreased ion levels.

In Table 17A the different amino acids were studied at the same external concentration of 1.0 mM. Since Na^+ influenced the affinity constant of binding to the carrier, the possibility was considered that the concentration of an amino acid relative to its K_M value could be an important factor in determining the quantitative effects of an altered ionic composition of the medium. In Table 17B the results obtained with the four amino acids studied at external concentrations equivalent to their K_M values are presented. As can be seen, the percentage inhibition at low K^+ concentration was approximately the same for all the amino acids. With a reduced external Na^+ concentration, AIB at its K_M value of approximately 2.5 mM, appeared to be affected to the greatest extent, although the percentage decrease did not vary significantly among the other amino acids. It is therefore quite difficult to clearly differentiate between

TABLE 17A

The Effects of an Altered Ionic Composition of the Medium on the Uptake of Several Amino Acids at the Same External Concentration

Amino Acid (radioactive)	Normal Medium (Control) $(A_c - A_f)$ mM	Low Na^+ medium $(A_c - A_f)$ mM	Per Cent Decrease	Low K^+ medium $(A_c - A_f)$ mM	Per Cent Decrease
ACPC	9.50	7.10	25	6.54	31
Glycine	12.70	8.91	30	7.93	38
AIB	9.33	5.81	38	6.45	30
L-Methionine	5.86	4.29	27	3.92	33

The incubation was carried out at 37°C as described in Section 4.1 and in "Materials and Methods". The concentration of Na^+ in the low Na^+ medium was 42 mM and choline chloride replaced the NaCl removed. The concentration of K^+ in the low K^+ medium was 1.5 mM and NaCl replaced the K^+ removed. A_c and A_f represent the concentrations of amino acid in the tissue and extracellular medium respectively. Each radioactive amino acid was present at an external concentration of 1.0 mM.

TABLE 17B

The Effects of an Altered Ionic Composition of the Medium on the Uptake of Several Amino Acids at Concentrations Equivalent to their K_m Values

Amino Acid (radioactive)	External Conc. (mM)	Normal Medium (Control) $(A_c - A_f)$ mM	Low Na^+ Medium $(A_c - A_f)$ mM	% Decrease	Low K^+ Medium $(A_c - A_f)$ mM	% Decrease
ACPC	6.0	29.47	25.07	15	23.69	20
Glycine	4.0	27.91	22.30	20	21.08	25
AIB	2.5	16.58	11.89	28	11.96	28
L-Methionine	3.0	10.71	9.21	14	7.48	30

Incubation conditions were the same as described in Table 17A.

the numerous entry sites in mouse pancreas on the basis of an altered ionic composition of the medium.

5.6 Amino Acid Uptake in the Presence and Absence of Na^+

Vidaver (193), studying transport in pigeon red cells, found no saturable uptake for glycine or alanine in absence of Na^+. Several tissues such as kidney cortex, rat diaphragm and Ehrlich ascites cells could not concentrate AIB when Na^+ was completely removed from the external medium (200, 205, 201). Other studies carried out in the absence of external Na^+ indicated that in Ehrlich ascites cells L-methionine was still accumulated (200), while in bone, glycine and AIB, but not L-proline or L-hydroxyproline were concentrated (82). These observations suggested that incubation of mouse pancreas in Na^+-free media might result in amino acids, which migrate via different pathways, showing differences with regard to their ability to establish concentration gradients. Preliminary experiments had indicated (Section 5.1) that ACPC could maintain a concentration gradient in the complete absence of external Na^+. The results obtained with several other amino acids are presented in Table 18. The steady-state uptake levels are shown for both normal and Na^+-free media. All the amino acids showed concentrative ability in the absence of Na^+. Even though the levels were greatly reduced from those obtained with a normal Na^+ medium, they were still higher than could be explained on the basis of simple diffusion. Of the five neutral amino acids studied, L-proline had the greatest difficulty in maintaining a concentration gradient in the absence of Na^+. A comparison of L-lysine uptake with that of the

TABLE 18

The Uptake of Several Amino Acids from Normal and Sodium-Free Incubation Media

Amino Acid	Amino Acid Uptake (μmoles/g tissue water)	
	in the presence of Na^+	in the absence of Na^+
AIB	10.33	2.69
ACPC	10.50	3.22
L-Methionine	6.86	2.34
Glycine	13.70	3.25
L-Proline	8.91	1.24
L-Lysine	3.51	2.64

The incubation was carried out at 37°C as described in Section 4.1 and "Materials and Methods". All amino acids were present at an external concentration of 1.0 mM. For the Na^+-free medium an equimolar concentration of choline was used and the medium was buffered with 10 mM tris chloride pH 7.4.

neutral amino acids indicated that this latter group was much more sensitive to sodium depletion than was the basic amino acid.

5.7 The Effects of Inhibitory Amino Acids on Amino Acid Uptake in the Presence and Absence of Na^+

Finerman and Rosenberg (82) studying transport in bone, found that in the presence of Na^+ the amino acids glycine, AIB, L-proline and hydroxyproline were mutually inhibitory, whereas in the absence of Na^+ glycine and alanine still inhibited AIB, but proline and hydroxyproline were without effect. They also noted that AIB, glycine and hydroxyproline inhibited L-proline uptake only in the presence of external Na^+ and not when it was omitted from the incubation medium. Similar work by Inui and Christensen (200) with Ehrlich ascites cells showed that AIB and L-methionine, which were mutually inhibitory in a normal Na^+ medium, would not inhibit each other in the complete absence of Na^+. These results suggested possible differences between the so called "Na^+-dependent" and "Na^+-independent" transport systems. Tables 19A and 19B show a similar study which was undertaken with mouse pancreas. The amino acids are placed in either of the two tables for comparison purposes only. These tables show the percentage decrease caused by the inhibitory amino acids in both normal Na^+ and Na^+-free media. In the case of ACPC uptake (Table 19A), AIB and L-methionine produced similar inhibitions in both media, while glycine was slightly more effective in the Na^+-free medium. With L-methionine as the transport substrate, greater inhibitions occurred in the normal Na^+ medium. In the case of L-tryptophan-3-C^{14}, AIB and ACPC were more effective

TABLE 19A

The Effects of Several Amino Acids on the Uptake of ACPC, L-Methionine and L-Tryptophan from Normal and Na^+-free Incubation Media

Inhibitor	ACPC Uptake ($A_c - A_f$)				L-Methionine Uptake ($A_c - A_f$)				L-Tryptophan Uptake ($A_c - A_f$)			
	Normal Na^+	% decrease	Na^+ free	% decrease	Normal Na^+	% decrease	Na^+ free	% decrease	Normal Na^+	% decrease	Na^+ free	% decrease
Nil	6.44	-	2.23	-	5.09	-	1.39	-	6.19	-	1.48	-
AIB	3.13	51	1.22	45	2.73	46	1.22	12	4.61	25	1.37	7
Glycine	3.82	41	0.95	57	2.71	46	1.00	28	5.48	11	1.22	18
L-Methionine	1.96	69	0.52	77	-	-	-	-	-	-	-	-
ACPC	-	-	-	-	2.63	48	1.38	0	4.10	34	1.27	14

The incubation were carried out at 37°C for 60-90 minutes as described in "Materials and Methods" and Section 4.1. In the Na^+-free experiments choline and 10 mM tris chloride buffer pH 7.4 were used. All radioactive amino acids were present at an external concentration of 1.0 mM and all non-radioactive amino acids (inhibitors) were present at an external concentration of 10.0 mM. A_c and A_f are the tissue and extracellular amino acid concentrations expressed as millimolarities.

TABLE 19B

The Effects of Several Amino Acids on the Uptake of Glycine, L-Proline and AIB from Normal and Na^+-free Incubation Media

Inhibitor	Glycine Uptake ($A_c - A_f$)				L-Proline Uptake ($A_c - A_f$)				AIB Uptake ($A_c - A_f$)			
	Normal Na^+	% decrease	Na^+ free	% decrease	Normal Na^+	% decrease	Na^+ free	% decrease	Normal Na^+	% decrease	Na^+ free	% decrease
Nil	10.75	-	2.03	-	7.83	-	0.42	-	10.61	-	1.42	-
AIB	6.01	44	1.35	33	-	-	-	-	-	-	-	-
Glycine	-	-	-	-	3.02	61	0.15	64	3.20	70	0.67	53
L-Methionine	5.31	51	0.56	72	1.54	80	0.37	12	2.10	80	0.32	77
ACPC	6.51	39	1.42	30	2.53	67	0.14	67	3.35	69	0.85	40

Incubation conditions were as described in Table 19A.

in the Na^+ containing medium than in the Na^+-free medium. Glycine uptake (Table 19B) showed a slightly greater sensitivity to L-methionine inhibition in a Na^+-free medium, while L-proline accumulation was inhibited by glycine and ACPC to the same extent in both media. L-Methionine caused a much greater decrease in the transport of L-proline from a normal medium than from a Na^+-free medium. With AIB as the transported amino acid (Table 19B), L-methionine was just as effective in both media, whereas glycine and ACPC exerted a slightly greater effect in the normal Na^+ containing medium. These results indicate that there are several differences in amino acid interaction in a Na^+-free medium as compared with a normal Na^+ medium. Most probably these differences, when obtained, reflect an effect of Na^+ on the affinity of the competitive inhibitor for the site or sites involved in the transport process.

5.8 The Effects of Temperature and a Nitrogen Atmosphere on Amino Acid Uptake

Table 20 shows the results of a study of the uptake of three neutral amino acids and 1 basic amino acid under conditions of a nitrogen atmosphere and decreased temperature. The influence of these factors at normal Na^+ and in the absence of Na^+ was studied. Each of the neutral amino acids is representative of a separate transport system as demonstrated by the work of Bégin and Scholefield (48). Incubation in the presence of a 100% nitrogen atmosphere at 37°C gave lower neutral amino acid accumulation levels in both normal and Na^+-free media as compared to similar media gassed with pure oxygen (Tables 18 and 20). In the case of the three neutral amino acids, gassing with nitrogen produced

TABLE 20

The Effects of Decreased Temperature and a Nitrogen Atmosphere on Amino Acid Uptake from Normal and Na^+-free Media

Amino Acid	Amino Acid Uptake (μmoles/g tissue water) in a nitrogen atmosphere		Amino Acid Uptake (μmoles/g tissue water) at 15°C	
	Na^+-free medium	Normal medium	Na^+-free medium	Normal medium
ACPC	2.55	4.47	3.45	4.21
Glycine	2.67	4.84	2.27	4.47
L-Methionine	1.97	3.14	3.37	4.38
L-Lysine	2.60	2.37	1.90	1.88

In all instances the incubation was carried out for 60 minutes with a 1.0 mM concentration of external amino acid as described in "Materials and Methods". For the Na^+-free experiments, the removed sodium was replaced by an equimolar amount of choline and the sodium phosphate buffer was replaced by a tris chloride buffer pH 7.4. In the nitrogen atmosphere experiments, the flasks were gassed with pure nitrogen for 4 minutes and then incubated at 37°C. In the decreased temperature experiments, the flasks were gassed with pure oxygen for 4 minutes and then incubated at 15°C.

higher uptakes from the normal Na^+ medium than from the Na^+-free medium, whereas L-lysine uptake was quite similar in both types of media. Reduction in the temperature from 37°C to 15°C similarly resulted in higher uptakes from the Na^+ containing medium than from the Na^+-free medium for the three neutral amino acids. However, L-lysine accumulation was almost identical in both media. This indicates basic differences between the transport systems for L-lysine and those for the neutral amino acids.

5.9 The Effects of DNP and Ouabain on Amino Acid Uptake in the Absence of Na^+

Earlier studies by Bégin and Scholefield (46) with a normal Na^+ medium had shown that the addition of 0.04 mM ouabain caused very large reductions in amino acid accumulation i.e.> 50%. Furthermore, this system was also affected by small amounts of DNP. The effects of DNP and ouabain on the accumulation of several amino acids from a Na^+-free medium are shown in Table 21. Increasing the concentration of DNP from 0.08 mM to 0.4 mM caused a further reduction in the uptake, although even at this latter concentration of DNP, movement against a concentration gradient could be obtained. Ouabain concentrations as high as 1.0 mM did not appear to have any effect on the uptake of either of the two amino acids examined. Although the effectiveness of DNP indicated that the transport system, even in the absence of external Na^+, was dependent upon a source of metabolic energy, the ineffectiveness of ouabain suggested that the linkage between the Na^+- K^+ pump and metabolite transport might no longer be functional.

TABLE 21

The Effects of DNP and Ouabain on the Uptake of Several Amino Acids from a Na^+-free Medium

Additions to the medium	ACPC Uptake (μmoles/g tissue water)	L-Lysine Uptake (μmoles/g tissue water)	Glycine Uptake (μmoles/g tissue water)
Nil	3.13	2.65	3.15
0.08 mM DNP	3.10	2.48	2.60
0.4 mM DNP	2.34	2.22	2.16
1.0 mM ouabain	-	2.63	3.13

The incubation was carried out at 37°C for 60 minutes as described in "Materials and Methods". Removed Na^+ was replaced as described in Table 18. All labelled amino acids were present at an external concentration of 1.0 mM.

5.10 The Effects of pH on Amino Acid Uptake in the Absence of Na^+

At a normal external Na^+ concentration, the uptakes of the neutral amino acids were relatively insensitive to pH, while the uptake of L-lysine, a basic amino acid, was quite sensitive (Chapter 4). If Na^+ only regulated the affinities of substrates for the carrier molecule, and if the same carrier was involved in both the presence and absence of external Na^+, then pH should have a similar effect in both media. Table 22 shows the effects of pH on the uptake of ACPC, a neutral amino acid and L-lysine, a basic amino acid. Decreasing the pH from 7.4 to 5.2 hardly affected the uptake of ACPC, whereas L-lysine uptake showed a much greater reduction than the neutral amino acid. The accumulation of L-lysine was slightly higher at pH 8.0 than at 7.4, agreeing quite favourably with the effect observed in Chapter 4 with a normal Na^+ medium. These results therefore support the view that the "Na^+-independent" and "Na^+-dependent" uptakes are not due to two separate carrier systems.

SUMMARY OF CHAPTER 5

(1) Reduction in either the external Na^+ or K^+ concentrations resulted in a decreased amino acid uptake.

(2) No cation could satisfactorily replace Na^+ in the transport process, whereas Rb^+ could function as a suitable replacement for K^+. Concentration gradients were obtained with all the cations used to replace Na^+ and K^+.

TABLE 22

The Effects of pH on the Uptakes of ACPC and L-Lysine from Na^+-free Media

pH	ACPC Uptake (μmoles/g tissue water)	L-Lysine Uptake (μmoles/g tissue water)
5.2	3.50	2.15
6.0	3.51	2.52
7.4	3.62	2.61
8.0	-	2.87

The incubation was carried out at 37°C for 60 minutes in a medium containing 1.0 mM radioactive amino acid and tris chloride buffer of different pH values. The sodium of the Krebs-Ringer medium was replaced by an equimolar concentration of choline. The above table shows the results of a typical experiment.

(3) Reduction of either the external Na^+ or K^+ concentration resulted in an increased apparent affinity constant and an unaltered maximum concentration gradient.

(4) One Na^+ ion/molecule of ACPC functioned as co-factor in the transport process.

(5) At low Na^+ or K^+ concentration, the uptakes of ACPC, glycine, AIB and L-methionine were decreased to approximately the same extent.

(6) All the amino acids maintained concentration gradients in the absence of external Na^+.

(7) Similar and dissimilar effects of inhibitory amino acids were obtained in normal Na^+ and Na^+-free incubation media.

(8) In a nitrogen atmosphere at 37°C, the neutral amino acids gave higher accumulation levels in normal Na^+ than in Na^+-free media. The uptake of L-lysine was approximately equal in both media.

(9) In an 100% oxygen atmosphere at 15°C, the neutral amino acids gave lower uptake values in a Na^+-free medium than in a normal Na^+ medium. L-lysine uptake was the same in both media.

(10) In the complete absence of Na^+, amino acid uptake was inhibited by DNP and other amino acids, but not by ouabain.

(11) In the complete absence of Na^+, basic amino acid (L-lysine)

uptake was significantly affected by pH, while neutral amino acid (ACPC) uptake was relatively insensitive to changes in pH.

CHAPTER 6

STIMULATED AMINO ACID MOVEMENT IN MOUSE PANCREAS

6.1 Theoretical Considerations

In addition to transport and simple diffusion as basic factors involved in the movement of an amino acid across the cell membrane, there is the possibility that exchange diffusion may also modify the steady-state level attained within the cell. If a mixture of two amino acids is present inside and outside a cell membrane, then there are four possibilities for exchange diffusion. If exchange of a labelled amino acid such as A is being studied, then exchange of A with itself will have no effect upon levels of radioactivity or upon the chemical level of A on either side of the membrane. Effectively, therefore, this factor does not contribute to net movement. Similarly, exchange of B with itself will not be measured if only A is radioactive and as pointed out above homoexchange cannot alter the level on either side of the membrane. On the other hand, exchange of A inside the cell with B on the outside of the cell is a unidirectional process, the extent of which will be governed by a function involving the concentration of A inside the cell and of B outside of the cell. Similarly the exchange of B inside the cell with A outside the cell is another unidirectional process whose magnitude must depend upon a function involving the concentration of B inside the cell and of A outside the cell. There is no reason to believe that these two functions should be equal and opposite. If they are not, then this third factor of exchange dif-

fusion must be taken into account in all studies in which steady-state levels are involved. The work presented in this chapter is directed towards obtaining evidence for the process of exchange diffusion in mouse pancreas and examining several of the factors which control and influence this phenomenon. All fluxes reported are the mean of at least six determinations except where otherwise indicated.

6.2 Stimulated Amino Acid Uptake Into Mouse Pancreas

In preliminary studies involving only transport of amino acids into the pancreas, it had been noted that the interactions between L-tryptophan and L-methionine could not be accurately represented by the standard Lineweaver and Burk type of analysis. The initial experiments on the exchange process were therefore conducted with this pair of amino acids since it was felt that exchange diffusion phenomena might be the cause of this discrepancy. The entry of L-tryptophan into pancreatic tissue which was preincubated in the presence and absence of non-radioactive L-methionine is shown in Table 23. As can be seen, at each of the time intervals there is a greater accumulation of L-tryptophan into the tissue which had been preincubated with L-methionine. The difference between entry into "loaded" and "non-loaded" tissue (i.e. that due to exchange diffusion) increased progressively throughout the course of the incubation. Since transport and exchange diffusion occurred simultaneously, the incubation was carried out at 15°C to decrease the component due to active transport. As can be seen in the second column of Table 23, even at this lower temperature distribution ratios (inside/outside) as high as 3 could still be obtained. Therefore the values

TABLE 23

The Influx of Radioactive L-Tryptophan Into Mouse Pancreas Preincubated in the Presence and Absence of Non-Radioactive L-Methionine

Time in minutes	Uptake of L-Tryptophan (μmoles/g tissue water) when intracellular L-methionine is		△ (Exchange Diffusion)
	Absent	Present	
5	1.10	1.59	0.49
10	1.82	2.60	0.78
15	2.27	3.46	1.19
25	3.05	4.71	1.66

The pancreas was preincubated at 37°C for 60 minutes in the presence and absence of 2 mM non-radioactive L-methionine. The uptake of 1 mM radioactive L-tryptophan at 15°C was then measured as described in "Materials and Methods".

reported to be due to exchange diffusion in the influx situation are only approximate since they involve subtraction of quite large control values i.e. uptake into "non-loaded" pancreatic tissue.

6.3 The Influx and Efflux Portions of the Exchange Reaction

If the stimulated amino acid accumulation indicated in Section 6.2 was actually due to exchange diffusion and not simply to a decreased efflux of L-tryptophan caused by the presence of L-methionine intracellularly, then a stimulation of amino acid movement should be seen in both directions. Table 24 shows the results of a series of experiments whereby 5.4 mM L-methionine was present in the tissue and 1 mM L-tryptophan was in the external medium. The top half of the table shows the movement of radioactive tryptophan into methionine "loaded" and "non-loaded" cells and the bottom portion shows L-methionine exit into media in the presence and absence of external L-tryptophan. It is quite apparent from these results that not only can the presence of methionine inside the tissue lead to an increased extent of tryptophan uptake (in this case amounting to more than 30% at 15 minutes), but also that the presence of tryptophan in the medium can lead to a marked increase in the efflux of prepacked methionine from the pancreas (approximately double at 15 minutes). It is also apparent from these results that the fluxes attributable to exchange diffusion are of the same order of magnitude and that the total fluxes in and out are approximately equal. It should be noted that more accurate observations can be made when efflux by exchange is being measured since much smaller control blanks have to be

TABLE 24

The Exchange Between L-Tryptophan and L-Methionine in Mouse Pancreas at 15°C

Amino Acid Prepacked	Amino Acid in Medium	Direction of Radioactivity	Measurement of Radioactivity at		
			5 min.	10 min.	15 min.
Nil	L-Tryptophan*	In	1.45+	1.95	2.40
L-Methionine	L-Tryptophan*	In	1.85	2.60	3.27
L-Methionine*	Nil	Out	0.82	1.13	1.36
L-Methionine*	L-Tryptophan	Out	1.57	2.30	2.80

* Indicates the radioactive amino acid

\+ Expressed as μmoles of amino acid/g tissue water

The concentration of L-Methionine in the tissue was always 5.4 μmoles/g tissue water and the concentration of L-tryptophan in the medium was 1.0 mM.

Movement of radioactivity: (a) inward - The uptake of 1 mM radioactive L-tryptophan at 15°C into L-methionine "loaded" and "unloaded" tissue was followed as described in "Materials and Methods".

(b) outward - Pancreas was preincubated in the presence of 0.7 mM radioactive L-methionine for 60 minutes at 37°C and efflux at 15°C into new media was followed as described in "Materials and Methods".

subtracted. The technique employed in the study of efflux has the further great advantage that, in time course studies, successive aliquots of the medium may be taken for analysis while in studies of influx by exchange, individual samples of tissue have to be utilized.

6.4 Comparison of the Effects of Amino Acids on Transport and Exchange Diffusion

Stimulation of efflux of one amino acid through the presence of a second one in the incubation medium may also be interpreted in terms of an inhibition of reconcentration of the amino acid lost from the cell into the medium. This possibility seems to be disproved in the present studies by the demonstration that on incubation of pancreas, prepacked with methionine, the efflux of methionine is stimulated by the presence of tryptophan in the incubation medium,while the presence of methionine inside the cells increases the flux of tryptophan into the tissue i.e. that the increased flux occurs in both directions (Table 24). Even more conclusive proof that the process is one of exchange diffusion is apparent from the results presented in Table 25. The effects of 1 mM methionine and of 5 mM AIB on the transport of ACPC, and the ability of these amino acids to exchange with ACPC previously accumulated by the pancreas, are compared. The concentrations of AIB and L-methionine employed were chosen so that the two amino acids inhibited the uptake of ACPC to about the same extent. If the effects indicated in Table 24 were obtained simply because of an inhibition of reconcentration, then these two amino acids at these concentrations should have exactly

TABLE 25

The Effects of 5 mM AIB and 1 mM L-Methionine on Transport and Exchange Diffusion of ACPC

Movement of ACPC	Addition to External Medium		
	Nil	5 mM AIB	1 mM L-Methionine
Uptake	9.35	6.35	6.02
Exchange			
5 min at 15°C	0.73	0.73	1.28
15 min at 15°C	1.35	1.51	2.41

Uptake: The uptake of 1 mM radioactive ACPC in the presence and absence of 5 mM AIB and 1 mM L-methionine was measured at 37°C for 60 minutes as described in "Materials and Methods".

Exchange: The pancreas was preincubated in the presence of 0.5 mM radioactive ACPC at 37°C for 60 minutes to obtain a tissue concentration of 5.4 μmoles/g tissue water. Efflux of ACPC at 15°C into the various media was followed as described in "Materials and Methods".

the same effect on the rate of loss of ACPC from prepacked tissue. However, as shown in the lower part of this table, it is apparent that 5 mM AIB had almost no effect on the efflux of prepacked ACPC, but methionine at a concentration of 1 mM produced an increased efflux amounting to more than 75%. It can therefore be concluded that the increased flux of amino acid being studied in these experiments is due to exchange diffusion and that the process has its own specificity which is not necessarily the same as the specificity of the carrier system involved in the transport of amino acids.

6.5 The Effects of Increased Concentration on Stimulated Amino Acid Movement

Table 26 shows the effect of preloading the tissue with varying amounts of non-radioactive L-ethionine and then measuring the uptake of 1.0 mM radioactive L-methionine. As the extent of intracellular preloading increased, so did the portion due to exchange diffusion. With the highest concentration (16 mM in the preincubation medium) this amounted to an increase of approximately 67% over the control. In other experiments (not quoted) involving amino acid movement out of the tissue into media containing varying amounts of non-radioactive substrate, there was an increased efflux with increases in the extracellular amino acid concentration. Thus this concentration dependent flux can occur in both directions and would assume great importance in studies involving amino acid entry when there is a large concentration of inhibitor.

TABLE 26

The Effects of Increased Intracellular Preloading on the Uptake of L-Methionine - Methyl - C^{14}

Concentration of L-Ethionine in the Preincubation Medium (mM)	Uptake of L-Methionine-Methyl-C^{14} (μmoles/g tissue water)	Per Cent Increase Over Control
Nil	2.1	100
2	2.5	119
6	3.2	152
16	3.5	167

The preincubation was carried out at 37°C for 60 minutes as described in "Materials and Methods". The uptake of 1 mM L-methionine-methyl-C^{14} was then measured at 15°C for 10 minutes as described also in "Materials and Methods".

6.6 The Effects of the Length of Incubation on the Exchange Reaction

Johnstone and Scholefield noted that at low temperatures the homo-exchange of L-methionine in the Ehrlich cell was almost complete in 6 minutes (209) while hetero-exchange between L-ethionine and L-methionine reached a maximum level in approximately 10 minutes (73). Table 27 shows the results of a series of experiments measuring the efflux of glycine from pancreatic tissue at various time intervals into media containing no amino acid, 10 mM L-methionine or 10 mM L-valine. The increased efflux due to the presence of the external amino acid is shown in column 4 for L-valine and in column 6 for L-methionine. As can be seen, there is an increased efflux up to approximately 45 minutes with L-methionine and 60 minutes with L-valine. There is no significant change after this time. Therefore the exchange reaction does not go on indefinitely, but instead reaches a maximum stimulated level in a longer period of time than any previously reported. Values taken over the first 15 minutes of the incubation would closely approximate an initial rate of movement in the exchange reaction. It should also be noted that incubation for extremely long periods of time might result in an accumulation of the effluxing amino acid in the external medium, with the possibility of reconcentration becoming much more of a dominant factor.

6.7 The Effects of a Low External Na^+ Concentration on the Exchange Reaction

Studying the homoexchange diffusion of L-methionine in Ehrlich ascites cells, Johnstone and Scholefield (209) concluded that the exchange

TABLE 27

The Effects of Time on the Efflux Portion of the Pancreatic Exchange Diffusion Process

Time (mins.)	Efflux of glycine into an amino acid free medium (control)	Efflux of glycine into a medium containing 10 mM L-valine	△ over control	Efflux of glycine into a medium containing 10 mM L-methionine	△ over control
0	0	0	0	0	0
5	0.69	0.84	0.15	0.80	0.11
15	1.11	1.66	0.55	1.64	0.53
30	1.59	2.41	0.82	2.47	0.88
45	1.92	3.00	1.08	3.02	1.10
60	2.26	3.41	1.15	3.22	0.96
75	2.63	3.61	0.98	3.64	1.01
90	2.82	3.91	1.09	3.90	1.08
105	3.05	4.16	1.11	3.95	0.90
120	3.44	4.43	0.99	-	-

The pancreas was preincubated at 37°C in a medium containing 0.4 mM glycine-1-C^{14} for 60 minutes, resulting in an initial concentration of 5.3 μmoles of glycine/g tissue water. Efflux of glycine-1-C^{14} into the various media was followed at 15°C as described in "Materials and Methods".

Efflux is expressed as μmoles of glycine/g tissue water.

diffusion process was independent of the presence of either Na^+ or K^+. In contrast, Wheeler and Christensen (197) have reported that the countertransport process for L-alanine in rabbit red cells was dependent upon the presence of Na^+ in the external medium. A similar study was undertaken with mouse pancreas strips as shown in Table 28. The tissue was preloaded with radioactive ACPC in all cases and efflux was followed into normal media in the presence and absence of external L-methionine (first portion of the table) and into low Na^+ media in the presence and absence of external L-methionine (last 2 columns of the table). At each of the time intervals indicated, reduction in the external Na^+ content had no significant effect on efflux of ACPC, either in the presence or absence of an external amino acid. Similar experiments (not quoted) involving the addition of 1 mM ouabain to the external medium of the exchange system produced no significant changes. These results indicate that interference with the $Na^+ - K^+$ pump has no effect on the exchange diffusion process in mouse pancreas. In addition, other experiments (not quoted) with these same amino acids showed no significant effect of pH on this phenomenon.

6.8 The Specificity of the Exchange Diffusion Process

The results presented in Section 6.4 suggested that the exchange process had its own specificity which was not necessarily the same as the specificity of the carrier system involved in the transport of amino acids. This specificity is further exemplified by the results presented in the following tables which concern the ability of various

TABLE 28

The Effects of a Decreased External Na^+ Concentration on the Exchange Diffusion Process in Mouse Pancreas

Time (minutes)	ACPC efflux into a normal Na^+ medium containing		ACPC efflux into low Na^+ medium containing	
	Nil	1 mM L-Methionine	Nil	1 mM L-Methionine
2	0.19*	0.30	0.20	0.27
8	0.43	0.83	0.41	0.79
15	0.64	1.24	0.63	1.21

* Efflux is expressed as μmoles of ACPC/g tissue water

Pancreas was preincubated with 0.2 mM radioactive ACPC for 60 minutes at 37°C in order to obtain a tissue concentration of 2.3 μmoles of ACPC/g tissue water. The efflux of ACPC at 15°C into the various media was followed as described in "Materials and Methods". The low Na^+ medium contained 17 mM Na^+ and the decreased NaCl content was replaced by an equimolar concentration of choline chloride.

amino acids to stimulate the efflux of certain intracellular amino acids from mouse pancreas. Each table shows the efflux of amino acid at several time intervals. The exchange due to the presence of each substrate is expressed as per cent in excess of the control efflux. This value is based on the total efflux at the end of 15 minutes incubation. All values represent the mean of at least six determinations.

(A) The Effects of Amino Acids on the Efflux of Intracellular Glycine.

Table 29 shows the results of a series of experiments designed to examine the specificity of several amino acids in the exchange process with intracellular glycine. Taurine which was a poor inhibitor of glycine transport (Table 8) was also a poor exchanger. However, AIB and L-proline, two excellent inhibitors of glycine transport (Table 8), were also rather poor from the point of view of exchange capabilities. The physiological isomer GABA, although shown previously to be poorly concentrated by the pancreas, appeared to have a significant affinity for the system involved in the exchange process leading to a stimulated efflux of glycine. Homo-exchange was quite significant, although there were a number of amino acids which, when present in the external medium, exhibited slightly greater exchange ability than glycine. Leucine, although just as effective an inhibitor of glycine uptake as L-proline (Table 8), was more effective than L-proline in causing a stimulated efflux from glycine "loaded" pancreatic tissue. Of all the amino acids studied L-tryptophan and L-valine caused the greatest efflux, amounting to a 74% increase over the control.

TABLE 29

The Effects of Several Amino Acids on the Efflux of Glycine-1-C^{14} from Mouse Pancreas

Amino Acid added(5 mM)	Efflux (μmoles/g tissue water)			Percentage of Control*
	3 min.	9 min.	15 min.	
Nil	0.60	0.97	1.26	100
Taurine	0.60	0.99	1.25	100
AIB	0.59	1.05	1.34	106
L-Proline	0.75	1.42	1.72	136
GABA	0.89	1.45	1.89	150
L-Threonine	0.94	1.63	1.92	152
Glycine	0.74	1.53	2.01	160
ACPC	0.93	1.71	2.04	162
L-Methionine	0.72	1.38	2.04	162
DL-α-Amino-n-Butyric Acid	0.89	1.61	2.05	162
L-Leucine	0.85	1.50	2.08	165
L-Tryptophan	0.99	1.83	2.20	174
L-Valine	1.01	1.55	2.19	174

* Based on the total efflux at the end of 15 minutes.

Pancreas was preincubated with 0.5 mM glycine-1-C^{14} at 37°C for 60 minutes resulting in an initial tissue concentration of 6.6 μmoles of glycine/g tissue water. Efflux into media containing various amino acids was then followed as described in "Materials and Methods".

(B) The Effects of Amino Acids on the Efflux of Intracellular AIB

The results of this series of experiments are presented in Table 30. At these concentrations, homo-exchange of AIB amounted to a significant 21% over the control, although several other amino acids caused slightly greater effluxes. Amino acids such as glycine and L-ethionine, which were excellent inhibitors of AIB transport (Table 8), were quite poor as exchangers in this sytem. Although L-proline was a much more effective inhibitor of AIB transport than taurine (Table 8), these two amino acids stimulated AIB efflux to approximately the same extent. The maximum stimulated effluxes observed with L-tryptophan and L-leucine in the external medium, were only 34% and 39% respectively above the control. This represents approximately 53% of the maximal efflux obtained when glycine was present intracellularly (Table 29). The results clearly indicate that AIB, when present intracellularly, is not as effective in the exchange reaction as its structural analog glycine.

(C) The Effects of Amino Acids on the Efflux of Intracellular L-Proline.

Bégin and Scholefield (47) have shown that proline is transported into mouse pancreas only after two molecules of the amino acid have combined with adjacent sites on the surface of the carrier. In this manner L-proline is quite different from AIB and glycine discussed in the previous sections. The effects of several amino acids on the efflux of L-proline is shown in Table 31. Although each of the amino acids had

TABLE 30

The Effects of Several Amino Acids on the Efflux of AIB-1-C^{14} from Mouse Pancreas

Amino Acid added (5 mM)	Efflux (μmoles/g tissue water)			Percentage of Control*
	3 min.	9 min.	15 min.	
Nil	0.56	0.97	1.21	100
Glycine	0.81	1.06	1.29	106
L-Ethionine	0.61	1.08	1.40	115
ACPC	0.60	1.11	1.41	116
L-Alanine	0.57	1.12	1.44	119
AIB	0.65	1.18	1.47	121
L-Proline	0.83	1.32	1.49	123
Taurine	0.81	1.25	1.50	124
L-Threonine	0.72	1.26	1.53	126
L-Valine	0.76	1.27	1.57	130
L-Tryptophan	0.67	1.22	1.62	134
L-Leucine	0.88	1.45	1.68	139

* Based on the total efflux at the end of 15 minutes.

Incubation conditions were as described in Table 29 except that the preincubation was carried out in the presence of 0.5 mM AIB-1-C^{14} for 90 minutes, resulting in an initial tissue concentration of 5.4 μmoles of AIB/g tissue water.

TABLE 31

The Effects of Several Amino Acids on the Efflux of L-Proline-U-C^{14} from Mouse Pancreas

Amino Acid added (5 mM)	Efflux (μmoles/g tissue water)			Percentage of Control*
	5 min.	10 min.	15 min.	
Nil	1.01	1.48	1.63	100
L-Methionine	1.02	1.34	1.78	107
Glycine	1.12	1.61	1.81	111
ACPC	1.15	1.68	1.84	113
L-Tryptophan	1.05	1.50	1.84	113
AIB	1.05	1.47	1.92	118
L-Proline	1.28	1.59	1.95	120
L-Leucine	1.24	1.71	2.13	124

* Based on the total efflux at the end of 15 minutes.

Incubation conditions were as described in Table 29 except that the preincubation was carried out in the presence of 0.6 mM L-proline-U-C^{14} for 90 minutes, resulting in an initial tissue concentration of 5.8 μmoles of L-proline/g tissue water.

an affinity for the system involved in the exchange process leading to an efflux of L-proline, it was quite small as indicated by a maximum stimulation of efflux with L-leucine of only 24% above the control. The results clearly indicate that L-proline when present intracellularly is a slightly poorer exchanger than AIB and a much less effective one than glycine.

(D) The Effects of Amino Acids on the Efflux of Intracellular L-Methionine.

The specificity of L-methionine exchange is shown in Table 32. Amino acids such as AIB, L-proline and glycine, although good inhibitors of L-methionine uptake (Table 8), were extremely poor participants in the exchange reaction. GABA, though poorly concentrated itself, caused a significant stimulation of efflux amounting to approximately 66% over the control value. The amino acids ACPC and L-valine, previously shown to migrate by a system distinct from that of L-methionine, had significant affinity for the system involved in the exchange process leading to an increased efflux of methionine, as indicated by stimulated effluxes of 151% and 177% respectively above the control. In addition, the homo-exchange situation (with non-radioactive L-methionine in the external medium) showed a greater efflux than any of the twelve hetero-exchange possibilities. With a maximal stimulated efflux of 197% over the control, L-methionine, when present intracellularly, has a much greater exchange capacity than any of the amino acids discussed in the previous sections.

TABLE 32

The Effects of Several Amino Acids on the Efflux of L-Methionine-Methyl-C^{14} from Mouse Pancreas

Amino Acid added (5 mM)	Efflux (μmoles/g tissue water)			Percentage of Control*
	2 min.	8 min.	15 min.	
Nil	0.57	1.00	1.41	100
Taurine	0.53	1.01	1.41	100
AIB	0.53	1.11	1.57	111
L-Proline	0.61	1.18	1.62	115
Glycine	0.51	1.19	1.82	129
GABA	0.72	1.76	2.34	166
DL-α-Amino-n-Butyric Acid	0.70	2.01	2.92	207
L-Threonine	0.73	2.07	3.08	219
ACPC	0.99	2.83	3.53	251
L-Tryptophan	1.10	2.68	3.56	252
L-Cysteine	0.91	2.76	3.77	267
L-Leucine	1.21	2.98	3.81	270
L-Valine	1.01	2.88	3.91	277
L-Methionine	1.48	3.23	4.19	297

* Based on the total efflux at the end of 15 minutes.

Incubation conditions were as described in Table 29 except that the pre-incubation was carried out in the presence of 0.7 mM L-methionine-methyl-C^{14} for 60 minutes, resulting in an initial tissue concentration of 5.4 μmoles of L-methionine/g tissue water.

(E) The Effects of Amino Acids on the Efflux of Intracellular ACPC.

The results of this series of experiments are presented in Table 33. Just as in the case of L-methionine, the amino acids AIB, L-proline and glycine had the least ability to exchange with intracellular ACPC under the present experimental conditions. L-Threonine and L-leucine, good inhibitors of ACPC transport, were also excellent exchange partners as shown by stimulated effluxes of 85% and 82% respectively above the control. Here too GABA exhibited an affinity for the exchange system, although the value of 42% in excess of the control was slightly less than that seen with L-methionine intracellularly (Table 32). Methionine, which has been shown to enter by a transport system distinct from ACPC, had a very large affinity as indicated by a 174% increase over the control. Homo-exchange in this situation was also quite large (160% increase). This compares favourably with the observations made with the L-methionine exchanging system. The maximum possible stimulated efflux of 174% is much higher than that obtained when glycine, AIB or L-proline were present intracellularly.

(F) The Effects of Amino Acids on the Efflux of Intracellular L-Tryptophan.

The exchange specificity for L-tryptophan, an amino acid which enters the mouse pancreas by means of at least two distinct systems (see Chapter 4), was examined and the results are presented in Table 34. Similar to several of the previous amino acids, L-proline and taurine were extremely poor choices for exchange, while GABA had a significant affinity for

TABLE 33

The Effects of Several Amino Acids on the Efflux of ACPC-C^{14} from Mouse Pancreas

Amino Acid added (5 mM)	Efflux (μmoles/g tissue water)			Percentage of Control*
	2 min.	8 min.	15 min.	
Nil	0.43	0.93	1.35	100
Taurine	0.43	0.93	1.35	100
AIB	0.48	0.98	1.51	112
L-Proline	0.70	1.35	1.81	134
Glycine	0.45	1.07	1.88	139
GABA	0.61	1.31	1.92	142
L-Phenylalanine	0.58	1.41	2.08	154
L-Tryptophan	0.64	1.67	2.43	180
L-Leucine	0.56	1.74	2.45	182
L-Threonine	0.49	1.54	2.50	185
L-Serine	0.59	2.12	3.27	242
L-Cysteine	0.61	2.15	3.31	245
L-Valine	0.76	2.41	3.48	258
ACPC	0.70	2.48	3.51	260
L-Methionine	0.90	2.62	3.69	274

* Based on the total efflux at the end of 15 minutes.

Incubation conditions were as described in Table 29 except that the preincubation was carried out in the presence of 0.5 mM ACPC-C^{14} for 60 minutes, resulting in an initial tissue concentration of 5.4 μmoles of ACPC/g tissue water.

TABLE 34

The Effects of Several Amino Acids on the Efflux of L-Tryptophan-3-C^{14} from Mouse Pancreas

Amino Acid Added (5 mM)	Efflux (µmoles/g tissue water)			% of Control*
	3 mins.	9 mins.	15 mins.	
Nil	0.92	1.60	1.95	100
L-Proline	0.89	1.53	1.97	100
Taurine	0.90	1.59	1.99	102
GABA	1.14	2.04	2.88	148
ACPC	1.26	2.54	3.53	181
L-Tryptophan	1.41	2.92	3.75	192
L-Threonine	1.48	3.10	4.02	206
L-Valine	1.44	3.15	4.03	206
L-Methionine	1.31	3.08	4.26	218
L-Phenylalanine	1.91	3.45	4.29	220
L-Leucine	1.65	3.30	4.43	227

*Based on the total efflux at the end of 15 minutes.

Incubation conditions were as described in Table 29 except that the preincubation was carried out in the presence of 0.8 mM L-Tryptophan-3-C^{14} for 90 minutes, resulting in an initial tissue concentration of 6.5 µmoles of L-tryptophan/g tissue water.

the exchange system. With this particular amino acid homo-exchange was quite good (92% increase), although several other amino acids were slightly more effective in the exchange reaction. When present intracellularly tryptophan had a much greater exchange capacity than glycine, AIB or L-proline and was slightly less effective than L-methionine or ACPC.

(G) The Effects of Amino Acids on the Efflux of Intracellular L-Lysine.

The results presented in Chapter 4 indicated that the transport system serving for the entry of the basic amino acid L-lysine could also accommodate a number of neutral amino acids. The question arose as to whether L-lysine, when present intracellularly, would be a good exchange participant at concentrations similar to those used with the previous neutral amino acids. The results of these experiments are presented in Table 35. Although all of the amino acids produced varying degrees of stimulated efflux, the maximum effect was only 20% in excess of the control value. These results clearly indicate that L-lysine, when present intracellularly, behaves in a similar manner to AIB and L-proline in the exchange reaction.

6.9 Concentration Effects on the Exchange Reaction

In view of some previous anomalies, it was of interest to determine whether the concentration effects controlling exchange differed in any way from those controlling transport. The system investigated first was that in which ACPC inside the pancreas was exchanged with L-methionine

TABLE 35

The Effects of Several Amino Acids on the Efflux of L-Lysine-U-C^{14} from Mouse Pancreas

Amino Acid Added (5 mM)	Efflux (µmoles/g tissue water)			% of Control*
	4 mins.	8 mins.	16 mins.	
Nil	1.15	1.48	1.73	100
L-Phenylalanine	1.26	1.49	1.77	102
ACPC	1.30	1.73	1.93	111
L-Methionine	1.16	1.46	2.00	114
L-Leucine	1.23	1.58	2.00	114
Taurine	1.25	1.63	2.00	114
Glycine	1.39	1.76	2.03	117
L-Tryptophan	1.44	1.75	2.07	120
L-Proline	1.35	1.69	2.06	120
L-Valine	1.37	1.64	2.07	120

*Based on the total efflux at the end of 16 minutes.

Incubation conditions were as described in Table 29 except that the preincubation was carried out in the presence of 1.7 mM L-lysine-U-C^{14} for 60 minutes, resulting in an initial tissue concentration of 6.4 µmoles of L-lysine/g tissue water.

in the external incubation medium. These results are presented in Fig.10. In the first set of experiments shown in Fig.10A, pancreas was preloaded with ACPC to different levels and efflux was followed into external media in the presence and absence of 1 mM L-methionine. That portion due to exchange (difference between efflux in the presence and absence of external L-methionine over the first 5-15 minutes) at each of the ACPC concentrations is plotted against the concentration of ACPC in the tissue at the start of the incubation. It should be noted that these are only approximate values since they involve data in which large blank values have been substracted from the overall flux i.e. the rate of movement of ACPC into an amino acid free medium has been subtracted from the total flux observed in the presence of methionine. It is apparent that the system would only be termed saturated at extremely high levels of ACPC inside the cell. On the other hand, when the concentration of ACPC inside the cell was maintained at 10.5 mM and the concentration of methionine in the outside medium was varied the results shown in Fig.10B were obtained. This plot of exchange diffusion rate versus the concentration of external L-methionine indicates that the external amino acid is able to saturate the exchange system at quite low concentrations. Approximate estimates of an affinity constant have been obtained by plotting the data according to the method of Lineweaver and Burk (231) i.e. a double reciprocal plot of the efflux rate against the concentration of external amino acid varied. The value obtained for the affinity constant depended upon the period of time over which the rates were calculated, but they were always of the same order. For the other systems the experimental set-up

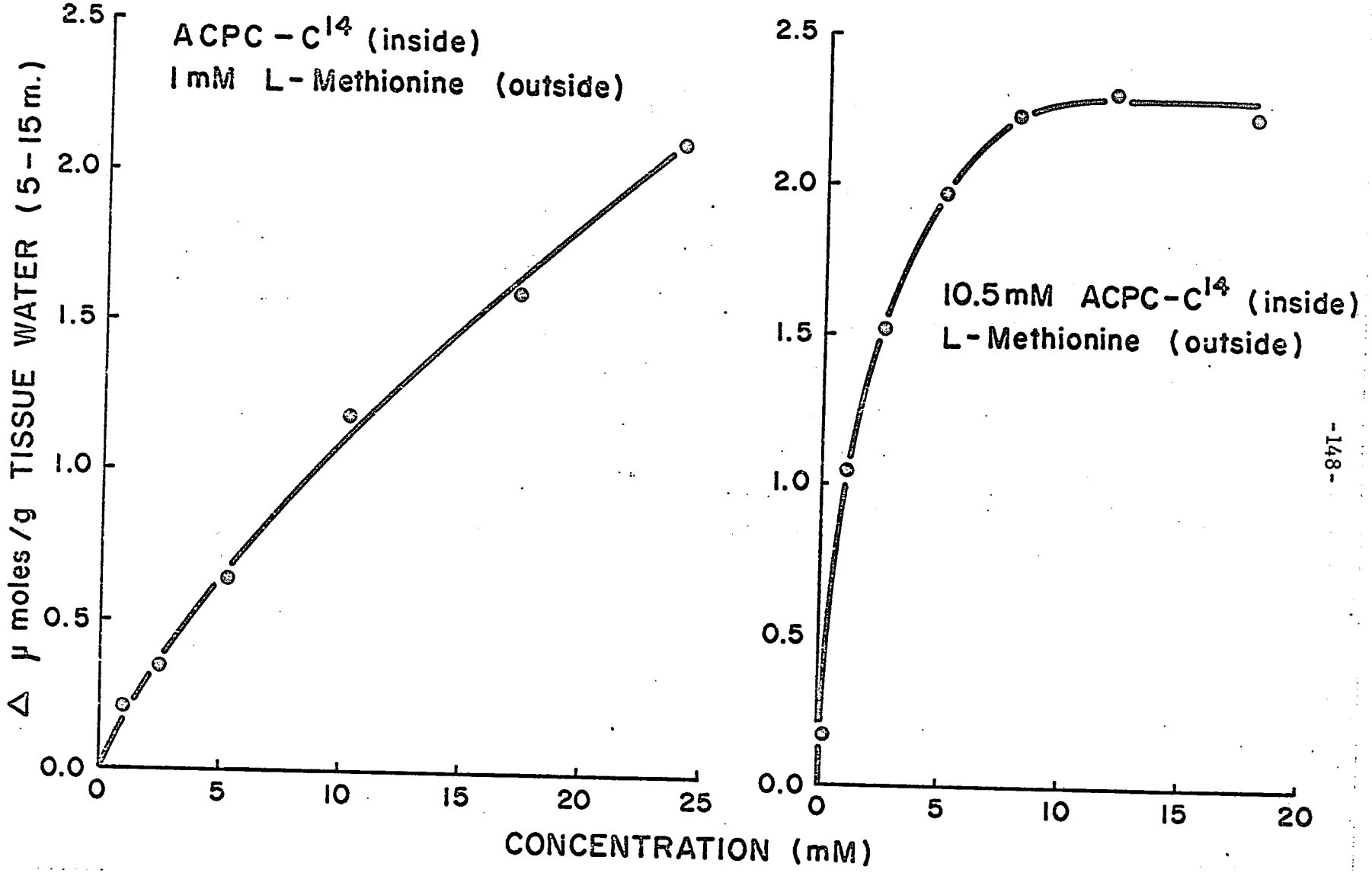

FIG. 10: The effects of variation in the concentration of amino acid (a) inside the tissue (ACPC) or (b) in the external medium (L-methionine) on the increased flux of ACPC from pancreatic tissue into an incubation medium containing L-methionine.
Incubation conditions were as described in the text and "Materials and Methods".

was similar to that shown in Fig.10B and a summary of some of the experimental affinity values obtained is presented in Table 36. In the examples where the concentration of methionine present in the outside medium was varied, the affinity constant was of the order of 2 mM and it is worth noting that the affinity constant characterizing the transport of methionine into mouse pancreas has previously been shown to be 2.9 mM (48). Similarly the uptake of ACPC is characterized by an affinity constant of 5.8 mM (48) which is of the same order as the constant characterizing the affinity of ACPC present in the medium for the system responsible for exchanging it with the prepacked methionine. It should be noted that the affinity constant for exchange was of the same order whether the external amino acid varied was in a homo-exchange or hetero-exchange situation (compare lines 2 and 4 of Table 36) or whether the concentration of intracellular amino acid was the same or not (compare lines 2 and 3 of Table 36).

Results of previous sections had indicated that amino acids such as AIB or glycine, although excellent inhibitors of the transport of most amino acids, had very little exchange diffusion capabilities. Such a situation could exist if the affinity constants characterizing exchange were much higher than those for transport i.e. decreased affinity during exchange. Table 37 shows the results of a series of experiments wherein the tissue concentration of AIB-1-C^{14} was kept constant and the concentration of non-radioactive AIB in the external medium was varied (first part of the table). Similarly the concentration of intracellular glycine-1-C^{14} was kept constant and the non-labelled glycine

TABLE 36

The Affinity Constants Characterizing the Exchange Diffusion Process in Mouse Pancreas

Amino Acid Inside the Pancreas	Amino Acid in the External Medium	Amino Acid whose concentration is varied	K_{ex} (mM) Affinity constant for exchange
ACPC	L-Methionine	$ACPC_{in}$	> 30
ACPC (5.4 mM)	L-Methionine	$L\text{-Methionine}_{out}$	1.5
ACPC (10.5 mM)	L-Methionine	$L\text{-Methionine}_{out}$	2.2
L-Methionine (5.5 mM)	L-Methionine	$L\text{-Methionine}_{out}$	3.0
L-Methionine (6.0 mM)	ACPC	$ACPC_{out}$	7.1
ACPC (5.4 mM)	ACPC	$ACPC_{out}$	5.0

The incubation conditions were as described in the text and in "Materials and Methods". $ACPC_{in}$ and $ACPC_{out}$ refer to ACPC inside the cell and ACPC in the medium respectively. $L\text{-Methionine}_{out}$ refers to L-Methionine in the external medium. In all cases the efflux of radioactive amino acid was followed.

TABLE 37

The Effects of an Increased External Amino Acid Concentration on the Homo-Exchange Diffusion of AIB and Glycine in Mouse Pancreas

Time (minutes)	Efflux of radioactive AIB into media containing			Efflux of radioactive glycine into media containing		
	8 mM AIB	14 mM AIB	32 mM AIB	10.14 mM Glycine	20.28 mM Glycine	30.42 mM Glycine
3	0.68*	0.68	0.68	0.68	0.79	0.74
9	1.22	1.16	1.01	1.29	1.60	1.49
15	1.43	1.52	1.35	1.77	2.12	2.06

*Efflux expressed as μmoles/g tissue water.

The pancreas was preloaded either with 5.4 μmoles of AIB/g tissue water or 6.5 μmoles of glycine/g tissue water. Efflux at 15°C into the various media was followed as described in "Materials and Methods".

present externally was varied. In both cases, the efflux of the radioactive amino acid was followed at the different time intervals indicated. If the affinity constant for exchange was higher (decreased affinity), then the efflux should progressively increase as the external amino acid concentration is elevated and this should continue up to very high external concentrations. But as can be seen from the results, there was no significant increase beyond 8 mM AIB externally in the first case and 20.28 mM glycine in the second case. This gave affinity constants for exchange which were quite similar to those characterizing the transport of these amino acids (48). This is in contrast to the Ehrlich cell where "the small driving of the outward migration by the short-chain neutral amino acids is nearly linear up to 70 mM whereas the K_m values for their uptake are 0.5 to 0.6 mM ..." (67).

6.10 The Effects of Extracellular Amino Acid Mixtures on the Exchange Reaction

The similarity of the affinity constant values strongly suggests that exchange systems may have much in common with transport systems and that competition or interaction between amino acids for the exchange system might occur just as it does in the case of the transport system. In Table 38 the fluxes of L-methionine, prepacked into the pancreas, have been studied in media containing non-radioactive L-threonine, L-methionine or a mixture of these two amino acids. The concentrations employed were those below the saturation level, to be certain that additive effects were possible. If competition for one exchange site occurred through the

TABLE 38

The Effects of Mixtures of L-Threonine and L-Methionine in the External Medium on the Efflux of L-Methionine-Methyl-C^{14} from Mouse Pancreas

Time (minutes)	Additions to External Medium		
	5 mM L-Threonine	1.4 mM L-Methionine & 5 mM L-Threonine	1.4 mM L-Methionine
2	1.15*	1.58	1.23
5	1.89	2.57	1.85
8	2.38	3.11	2.35
12	2.82	3.45	2.72
15	3.15	3.79	3.10

* Efflux is expressed as μmoles/g tissue water.

Pancreas was preincubated with 0.75 mM labelled L-methionine for 60 minutes at 37°C in order to obtain a tissue concentration of 5.3 μmoles/g tissue water. Efflux at 15°C into the various media was followed as described in "Materials and Methods".

presence of two amino acids at below the saturation levels, then a significant increase in the flux of L-methionine should be noticeable. As seen in Table 38 the presence of both amino acids in the incubation medium produced a greater effect than either one present alone. This additive effect clearly indicates that an interaction between amino acids does occur in the exchange system.

In view of the other possibility that inhibitory effects might occur, a similar experiment (Table 39) was carried out in which 1 mM ACPC, 5 mM AIB or a mixture of these amino acids at these concentrations, were present in the incubation medium. In this case the concentration of ACPC in the medium was 1 mM in order to have the minimal possible concentration of substrate which would show a significant exchange diffusion effect. The concentration of AIB was one which caused a very marked inhibitory effect on the accumulation of ACPC from a medium containing the amino acid at a concentration of 1 mM (see Table 25). As can be seen from the results presented in Table 39 there was no significant effect of AIB on the homo-exchange of ACPC under these conditions. Therefore, although AIB was an inhibitor of ACPC in the transport reaction, it did not appear to inhibit in the exchange process involving this same amino acid. A possible reason for this discrepancy will be presented in the discussion.

SUMMARY OF CHAPTER 6

1. Not only can the presence of L-methionine inside the tissue lead to an increased extent of L-tryptophan uptake, but in addition the presence of L-tryptophan in the medium can lead

TABLE 39

The Effects of Mixtures of ACPC and AIB in the External Medium on the Efflux of Radioactive ACPC from Mouse Pancreas

Time (minutes)	Additions to External Medium			
	N i l	1 mM ACPC	1 mM ACPC & 5 mM AIB	5 mM AIB
2	0.43*	0.63	0.71	0.42
5	0.73	1.26	1.25	0.70
8	0.93	1.64	1.74	0.93
12	1.19	2.10	2.22	1.15
15	1.35	2.52	2.48	1.32

* Efflux is expressed as μmoles/g tissue water.

Pancreas was preloaded with an initial concentration of 5.5 μmoles of ACPC/g tissue water as described in "Materials and Methods". Efflux at 15°C into the various media was followed as described in the text and "Materials and Methods".

to a marked increase in the efflux of prepacked L-methionine from the pancreas. The fluxes attributable to exchange diffusion (in and out) are of the same order of magnitude and the total fluxes (in and out) are approximately equal.

2. The possibility of reconcentration is rejected since 1 mM L-methionine and 5 mM AIB, which inhibited the uptake of ACPC to about the same extent, behaved quite differently in exchange diffusion with intracellular ACPC.

3. Exchange diffusion increased as the extent of intracellular preloading increased. Similarly, there was a progressive increase in the efflux of intracellular amino acid as the concentration of extracellular amino acid was increased.

4. Hetero-exchange (outward) of glycine with L-methionine and L-valine increased progressively for approximately 45 and 60 minutes respectively, after which time there was no significant change.

5. Exchange diffusion of amino acids in mouse pancreas was not dependent upon the external Na^+ concentration.

6. The relative ability of intracellular amino acids to exchange were (in decreasing order): L-methionine, ACPC, L-tryptophan, glycine, AIB, L-proline, L-lysine.

7. When present extracellularly, the short-chain neutral amino acids (glycine, AIB, L-proline, etc.) exchanged very poorly in all instances. Where there was a significant stimulated

efflux of prepacked amino acid, the long-chain neutral amino acids were excellent exchangers.

8. The exchange system could only be saturated at extremely high levels of amino acid inside the cell. On the other hand, the external amino acid was able to saturate the exchange system at quite low concentrations. In the latter case, the affinity constant for the exchange reaction appeared to be quite similar to the affinity constant characterizing the transport of the amino acid into the pancreas.

9. The presence of two amino acids (each below the saturation level) in the incubation medium produced a greater stimulation of efflux of prepacked amino acid than either one acting independently. Such a significant increase in the flux would be seen if there was competition for the one exchange site.

10. With ACPC intracellularly, a mixture of AIB (at a concentration which did not cause exchange, but which inhibited transport) and ACPC (at a concentration which caused homo-exchange) in the external medium gave the same result as that obtained with homo-exchange of ACPC i.e. although AIB was an inhibitor of ACPC in the transport reaction, it did not appear to inhibit in the exchange process involving this same amino acid.

CHAPTER 7

DISCUSSION

The Pancreas and Amino Acid Transport

The results presented in Chapter 3 confirm the fact that amino acids are concentrated to high intracellular levels in adult mouse pancreas *in vitro*. Amino acid accumulation in the newborn, however, was markedly decreased, while pancreas from adult mice weighing 20-40 g accumulated amino acids to about the same extent. This would indicate that the important factor was the age rather than the size of the animal. Such differences between newborn and adult animals have also been observed in studies with brain slices (76) and might be due to incomplete development of the amino acid transport systems. Alterations in such systems concurrent with organ development are not without precedent e.g. the changes shown by the red blood cell during maturation (197).

The lower uptake in the salivary gland than in pancreas is particularly interesting in view of the close similarity of structure between these two organs. This would suggest that the amino acid uptake pattern is most probably related to the particular function of the gland. Comparison of pancreas from several sources has not only shown mouse to have the most active transport system, but has also suggested that generally the smaller the experimental animal, the greater the ability of the pancreas to accumulate amino acids *in vitro*. This would be in accordance with the basal metabolic level of various mammals

being approximately proportional to the body surface area. Since the basal metabolism reflects the energy requirements for maintenance and conduct of fundamental cellular processes such as active transport, this might account for the generally lower amino acid accumulation in pancreas from larger animals.

Amino acid uptake was much lower in pancreas from mice bearing an Ehrlich ascites tumor than in pancreas from normal mice. However, other experiments have shown that the exchange diffusion process is unaffected.[1] The observed decrease might be due to an effect on energy levels, energy linkage to the transport system or on the $Na^+ - K^+$ dependent ATPase rather than to an actual loss of part of the membrane carrier, if it is assumed that the amount of carrier determines the amount of exchange. A great deal of work still remains to be done on the host-tumor aspect of the transport problem.

Steady-State Amino Acid Accumulation in Mouse Pancreas

The non-linear Lineweaver and Burk plot for the uptake of L-tryptophan is the first positive evidence that a dual mode of entry may exist in the pancreas. This type of analysis suggests that at least two distinct systems operate. Similar findings have also been reported for the entry of glycine into reticulocytes (84) and L-lysine into kidney cortex slices (121). Although the other amino acids gave a linear Lineweaver and Burk plot, this is not conclusive evidence in favor of a single system or site, since non-linearity would be observed only if the K_m values were separated by at least one order of magnitude (234).

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[1] Clayman, S. and Scholefield, P.G., unpublished observations.

In addition, the affinity constant for L-lysine uptake into mouse pancreas is much greater than that of any of the other amino acids previously studied (46-48). In spite of the linearity of the Lineweaver and Burk plot, the high K_m value could well be an indication of a double mode of entry. Recent work (121) has suggested that there are two routes for L-lysine uptake into kidney cortex slices, although the second transport system (with a small K_m value) was only apparent at much lower substrate concentrations than those used in the present experiments.

In kidney there is a separate transport system for basic amino acids (42, 115, 235) which overlaps that for neutral amino acids to a slight extent. Such a system may be present in the intestine (161, 169, 236) but it is not as well defined. Mutual inhibition during transport occurs not only among the basic amino acids themselves, but also between the basic and neutral amino acids (160, 169, 236). Similar work (72) with brain slices has suggested that separate systems are involved in the transport of the short and long chain basic amino acids. A particular amino acid seemed to have an affinity for more than one transport site - a high affinity for its primary site of transport and a lower affinity for a secondary site of entry. The present experiments show: (a) a significant effect of pH on L-lysine uptake which was not observed with the neutral amino acids, (b) a much greater effect of Na^+ removal on neutral amino acid uptake than on L-lysine uptake, (c) approximately the same uptake of L-lysine from normal and Na^+-free media when the incubation was carried out in a nitrogen atmosphere at 37°C or in an oxygen atmosphere at 15°C, and (d) no inhibition of L-lysine uptake by GABA or

taurine even at a 60-fold excess of inhibitor over substrate, although such inhibition was seen with the neutral amino acids. There may be a separate transport system in mouse pancreas for the basic amino acids (e.g. L-lysine), though the neutral amino acids would have a large affinity for this system. In addition, a sufficient concentration of the basic amino acids could eliminate either a large or small portion of the neutral amino acid uptake, depending upon the substrate under consideration. The affinity was highest for the ACPC transport site, while quite low for the methionine and glycine sites. The extent of inhibition of ACPC uptake by L-lysine is in contrast to that seen in the Ehrlich cell where excess lysine could eliminate only a clearly defined portion of the uptake of the neutral amino acids (149). It seems that in mouse pancreas there is much greater overlap among amino acids possessing neutral and cationic side-chains.

Blasberg and Lajtha (72) have reported that there are two separate sites for the uptake of small and large neutral amino acids in brain slices. Further, Oxender and Christensen (67, 74) have proposed that there are two distinct systems operating in the Ehrlich cell - one for short-chain and the other for long-chain neutral amino acids. A more extensive system has been suggested for mouse pancreas with separate transport sites for ACPC-valine-MeACPC, glycine, methionine-ethionine, and proline (48). The present findings indicate that AIB entry is at a separate site from those previously described. Thus, in mouse pancreas, there are at least two sites serving for the entry of short-chain neutral amino acids as well as at least two sites for long-chain neutral amino

acids. Furthermore, the relative insensitivity of the uptake of neutral amino acids to pH change and the similarity of the inhibition by DNP suggests that these sites may all exist on the same carrier molecule. Thus reference might be made to a "neutral amino acid transport system" possessing several different sites. Such a proposal is further supported by the very extensive interaction between neutral amino acids during the transport process as well as by the similarity of the percentage decrease in amino acid accumulation in the complete absence of external Na^+.

Of the amino acids studied thus far, GABA is the most poorly concentrated. The extremely low intracellular accumulation and the short period of time required to reach a steady-state level suggests that the major portion of uptake is probably via a diffusion-like phenomenon. This is in contrast to brain (72) where GABA is accumulated to extremely high intracellular levels in vitro. The inhibition of neutral amino acid uptake by GABA would indicate some affinity of GABA for the various sites involved in neutral amino acid transport.

Ions and Amino Acid Transport in Mouse Pancreas

The results presented in Chapter 5 clearly show that the accumulation of amino acids by the pancreas is influenced by changes in the ion composition of the external medium. Such decreases in uptake were seen only with changes in the external Na^+ and K^+ concentrations, since Ca^{++} and Mg^{++} have no effect in this system (46). Lineweaver and

'Burk analysis of amino acid uptake at low Na^+ concentrations showed a definite increase in the K_m value, although the maximum concentration gradient remained unaltered. This is in agreement with the results of Crane (189, 190) for sugar transport in the intestine, as well as those of Kipnis and Parrish (239) for AIB transport in rabbit lymphnodes and Cotlier and Beaty (206) for amino acid transport in the eye lens. They are, however, in contrast to the work of Christensen and his collaborators (196, 197, 200) who found that in pigeon erythrocytes, Ehrlich ascites cells and rabbit red cells, a change in the external Na^+ concentration resulted in both an altered K_m and V_{max} value.

Thus exogenous Na^+ affects the binding of substrate to the membrane carrier. This interaction is only enhanced by the presence of ions, as indicated by accumulation of amino acids against a concentration gradient in the absence of Na^+. Moreover, although the ternary complex (Na^+-carrier-amino acid) is required for high levels of active transport, it may come to be formed more via Na^+-carrier as the kinetically preferred route than by way of carrier-amino acid as the Na^+ concentration is raised. The ternary complex would constitute an additional mode of entry for Na^+, since the Na^+ would probably be released in the interior of the cell once the carrier is inactivated.

The suggestion of a separate cation site on the carrier could be adopted for this system, with the ions being regarded as "competitive activators" of the transport sites. The statement of Curran et al. (237) that "the first reaction cannot be association with Na^+ rather than the

amino acid since under these conditions, there would be no flux of amino acid in the absence of Na^+", could be avoided if the Na^+ and amino acid attached to their respective sites simultaneously. Such binding to two separate sites on the carrier surface would be needed in the pancreas since the existence of complexes such as carrier-Na^+-amino acid or carrier-amino acid-Na^+ would not be in agreement with the results obtained. If Na^+ and amino acid must bind to each other, then the uptake of amino acid would be inhibited by increasing the concentration of either Na^+ or amino acid (while holding the other at a constant concentration) since the Na^+ or amino acid would compete with the Na^+-amino acid complex for the subsequent binding to the carrier. Begin and Scholefield (47) have shown that the entry of L-proline involves attachment to two sites. It is possible that Na^+ may influence both proline sites during the transport reaction. Such a situation might require two cation sites on the carrier molecule, although the present data is insufficient to confirm this hypothesis.

Table 16 shows the inability of several ions to replace external Na^+ or K^+ adequately. However, the steady-state amino acid accumulation in the presence of these ions would be in agreement with the proposal that other metal ions can interact with the cation binding site on the carrier (238) although unable to function as efficiently. The maintenance of a normal amino acid uptake when K^+ is replaced by Rb^+ is in agreement with the findings of Riggs et al. (181) for glycine uptake in the Ehrlich cell, suggesting basic similarities between normal and neoplastic transport systems.

Vidaver (195) is in favor of a Na^+ gradient being responsible for amino acid movement, while Kipnis and Parrish (239) have considered that it is not simply the electrochemical gradient but also the "absolute level of Na^+ on both the inner and outer cell surface", that is primarily important. The present evidence indicates that in a mouse pancreas a decrease in the external Na^+ concentration results in impaired amino acid transport i.e. there is a dependence on the absolute level of Na^+ as well as on the Na^+ gradient.

Several investigators have obtained an approximate value for the number of Na^+ involved in the amino acid entry process. For instance, 1 Na^+ was required for amino acid uptake in diaphragm (239), two Na^+ for glycine uptake in rabbit red blood cells (197), two Na^+ for glycine uptake in pigeon erythrocytes (193) and one Na^+ for AIB uptake in the eye lens (206). The present findings are in favor of a single Na^+, rather than 2 Na^+, functioning as co-factor in the transport process in mouse pancreas. This evidence is by no means conclusive. It is possible that the reaction constants for 2 Na^+ may be so related that only reaction with the first Na^+ is kinetically observable, as suggested recently by Wheeler and Christensen (241) with the red blood cell. Such a requirement would not be applicable to concentrative accumulation by the pancreas in the absence of Na^+. Furthermore, it is not known whether the need for two ions indicates that 2 Na^+ operate through one cation site or whether two different cation sites each require the presence of 1 Na^+ in order to function.

The Na^+-insensitive uptakes of AIB and MeAIB in the Ehrlich cell

were not concentrative, while L-methionine uptake was concentrative (200). In leucocytes (198, 199), AIB, glycine, proline and alanine required Na^+ for transport, while ACPC and L-methionine were not Na^+-dependent. Similar work with bone (82) indicated that in the absence of Na^+, glycine and AIB were still accumulated while the entry of L-proline and hydroxyproline occurred via a non-mediated process which was similar to diffusion. Mouse pancreas is unique since concentration gradients could be obtained with all the neutral amino acids, regardless of whether they possessed a small or large side-chain. In this respect, the various transport carrier sites in mouse pancreas would be more analogous to the L-system of the Ehrlich ascites cell (67, 74). L-Proline, the only possible exception, would more closely correspond to the A grouping, although its mode of entry is quite different from that of amino acids in the Ehrlich cell (47).

The effect of DNP on amino acid accumulation in a Na^+-free medium is evidence that energy is still required for the Na^+-independent transport process. Jacquez and Sherman (8) have suggested that the linkage of energy to the transport system in the Ehrlich cell is such as to either increase the rate of outward movement of free carrier across the cell membrane, or increase the rate of dissociation of carrier-amino acid complex at the inner surface of the membrane. Since ouabain had no effect on amino acid accumulation at a concentration which was sufficient to interfere with the cation pump mechanism, it would appear that amino acid transport was not entirely dependent on an active Na^+- K^+ pump. This is in agreement with the observation that ouabain (1×10^{-5}M) caused a significant

'inhibition of Na^+ transport in the intact diaphragm without affecting AIB transport (205).

The presence of two independent and parallel pathways for amino acid influx, one Na^+-dependent and the other Na^+-independent, is unlikely since the maximum influx in the Lineweaver and Burk plot was the same in the presence and absence of external Na^+. If two independent pathways did exist, then the maximum influx would be additive, which was not the case. The similarity between the effect of pH on amino acid uptake in the presence and absence of Na^+ would also be in favor of the same carrier molecule participating in both processes.

Exchange Diffusion in Mouse Pancreas

In addition to transport and simple diffusion, there is also a flux of amino acids in mouse pancreas via an exchange reaction. The stimulated efflux of prepacked amino acid is definitely not due to failure to reconcentrate as suggested by Winkler and Wilson (113) on the basis of studies of galactoside transport in E. Coli. Johnstone and Quastel (214, 215) have concluded that the same amino acid carriers are involved in both transport and exchange diffusion in the Ehrlich cell. The present findings of a relative insensitivity to pH change (neutral amino acids) and a similarity between affinity constants in both phenomena are additional evidence in favor of such a hypothesis. Furthermore, additive effects of two amino acids were observed not only during transport (Table 12) but also in the exchange situation (Table 38).

Such interactions for one site would be expected if the same carrier molecule participated in both reactions.

The exchange reaction in mouse pancreas showed an influx to efflux ratio of about 1:1.6 rather than the theoretical 1:1 value. This can be accounted for on the following basis: in outward exchange, the movement into amino acid free media is substracted from the stimulated efflux in the presence of an amino acid and this difference is assumed to be due to exchange. Here the control represents diffusion and no difficulty is encountered. However, influx in the absence of intracellular amino acid is subtracted from the stimulated influx in the presence of intracellular amino acid and this is also presumed to be due to exchange. In this latter case the control value represents both diffusion and transport. Since the exchange and transport processes compete for the same carrier (8), less carrier will be available for exchange if transport occurs simultaneously. Therefore the value attributed to inward exchange would probably represent a minimum rather than the true value.

Bégin and Scholefield (48) have proposed that inhibition during transport occurs by attachment of the inhibitor to the substrate site with no subsequent translocation of the inhibitor. As indicated in the present studies, these "inhibitory" amino acids can exchange with intracellular amino acids which are normally transported by a different site i.e., there is an actual increase in the entry of the extracellular amino acid. It is possible that the inhibitor by attachment to some of the substrate sites, could be translocated during the transport reaction. This would involve amino acid entry at a secondary site as well as at a primary site.

If such is the case, then similarly in exchange diffusion, displacement of the intracellular amino acid from the carrier site could result in the same type of conformational change required for inward movement of the extracellular amino acid. Alternatively in the transport reaction, the presence of the attached substrate may result in non-translocation of the inhibitor as suggested by Bégin and Scholefield (48). Furthermore, after the exchange displacement reaction has occurred, the extracellular amino acid would be the only metabolite attached to the carrier. Thus translocation could take place i.e. as opposed to transport where both substrate and inhibitor are present and where there is no translocation. Although both situations are possible, the former (inhibitor translocation during transport and exchange) appears to be most likely.

In exchange diffusion the amino acid-carrier complex may proceed from BC to AC (where A and B are different amino acids) via free carrier. The similarity of the affinity constants for exchange and transport suggests that in exchange diffusion the amino acids may react with the free carrier, unless of course the affinity for the carrier-amino acid-complex is the same as for the free carrier. During the exchange process no free carrier may exist, if the carrier-amino acid complex at the outer membrane would also allow binding of the extracellular amino acid to another site on the molecule. In this way the carrier, with both amino acids attahced, may be non-functional. Release of the first amino acid could render the carrier-amino acid complex functional again (allosteric effect) and subsequently able to reorientate inward.

An important factor in exchange diffusion is that of lipid solubility. The poorly exchanging short-chain neutral amino acids are relatively lipid insoluble compared with the long-chain amino acids. Thus the effective concentration in the region of the carrier-amino acid complex may be much less than predicted on the basis of the extracellular amino acid concentration. An increase in the external amino acid concentration would result in a larger quantity of substrate in the membrane area to participate in the exchange reaction. Alternatively, the poor exchange ability of glycine, AIB and L-proline may involve difficulty in displacing amino acids from the carrier-amino acid-complex.

Since these poor exchangers were generally excellent inhibitors of the transport of most amino acids studied, the possibility also had to be considered that inhibition during transport was due solely to allosteric interference by the presence of the inhibitor at a second site, rather than competition for the same site. Such a possibility is disproved in the present studies, since a linear Dixon plot was obtained. Although no conclusive evidence is available at present, a situation which involves both "true" and "partially" competitive inhibition must also be considered. During transport the inhibitor would bind to both the substrate site and its own adjacent site. However, only that portion of the inhibitor which is attached to the substrate site will be capable of exchanging and so the stimulated efflux will be less than predicted on the basis of the transport studies. This type of interaction remains a definite possibility in view of the recent work of Alvarado (240) with sugar transport in the hamster small intestine. Here the carrier was

assumed to possess two different sites i.e. a sugar and phenol binding site. It was shown that phloretin binds only to the phenol site (allosteric inhibitor) while phlorizin, which gave a linear Thorn plot, binds simultaneously to both of these sites, thus being both a "true" and "partially" competitive inhibitor.

Oxender and Christensen (67, 74) have grouped glycine, AIB and L-proline together in the Ehrlich cell as very poor exchangers. Although AIB and L-proline in pancreas are in agreement with this classification, the maximum stimulation of 74% over the control obtained with glycine intracellularly would exclude it from this grouping. This is also in contrast to bone where glycine showed neither homo- nor hetero-exchange (82). With the pancreas, the long-chain amino acids when present intracellularly were better exchangers than the short-chain amino acids. It would appear that the intracellular amino acid determines the exchange capacity of the system, since with the intracellular short-chain amino acids AIB and L-proline, all external amino acids (both normally excellent and poor exchangers) were equally poor.

Bégin and Scholefield (48) have designated separate transport sites for ACPC, glycine and L-methionine based on kinetic studies. The exchange data provides additional evidence that a separate site exists for glycine since it behaves differently from ACPC or L-methionine (i.e. excellent exchangers). On this basis, ACPC and L-methionine could not be clearly differentiated. In addition, the AIB site (i.e. poor exchanger) could be distinguished from those serving for ACPC and L-methionine exchange (i.e. excellent exchangers).

The results presented in Chapter 6 indicate that although 5 mM AIB was a significant inhibitor of the transport of 1 mM ACPC, there was no inhibition by AIB of the homo-exchange of ACPC. In the transport situation it is very likely that this excess of AIB is not present in the area of the exchanging amino acid-carrier complex i.e. there is ACPC attached to the carrier intracellularly and extracellular ACPC diffusing towards the complex. Thus, at the existing inhibitor to substrate ratio, an inhibition may not be observed. Alternatively, another possibility must also be considered. The observed effect during transport may be interpreted primarily in terms of the "competitive" nature of the amino acid interaction. A result which is kinetically identical with competitive inhibition does not necessarily mean that the inhibitor competes with the substrate for attachment to one specific site on the enzyme surface. The kinetic equations only imply that the inhibitor and the substrate molecules cannot be attached to the enzyme at the same time. If ACPC and AIB, for example, occupied adjacent sites (i.e. completely different sites as suggested by the present studies) and if there was an extensive overlap, then it is possible to envisage conditions where the attachment of AIB would prevent the simultaneous attachment of ACPC and vice versa. The kinetic analysis of this type of interaction would be indistinguishable from a situation in which ACPC and AIB competed with each other for one single site during transport. If such is the case, then homo-exchange of ACPC would occur through its own site and would not be subject to inhibition by the presence of AIB.

Exchange diffusion of amino acids in the pancreas was not

dependent upon the external Na^+ concentration. This is in agreement with the findings of Johnstone and Scholefield (209), with the Ehrlich cell, but in disagreement with the Na^+-dependent L-alanine counter-transport seen in the rabbit red blood cell (197). In addition, the similarity between the affinity constants for the transport and exchange processes in mouse pancreas are in contrast to the quite striking differences between these two values in the red blood cell (228). This would suggest that there may be basic differences between the exchange diffusion and counterflow phenomena.

CLAIMS TO ORIGINAL RESEARCH

(1) Mouse pancreas accumulated amino acids to a greater extent than rat, guinea pig, dog or human (tumor) pancreas. Prior fasting (72 hours) did not have any effect on amino acid accumulation by mouse pancreas.

(2) Pancreas from adult mice showed a greater amino acid accumulation than pancreas from newborn.

(3) Kinetic analysis of L-tryptophan uptake suggests that at least two sites are involved.

(4) L-Lysine, which gave a linear Lineweaver and Burk plot over the present range of substrate concentrations, had a quite low affinity for its own transport system i.e. extremely high K_m value.

(5) In addition to those sites serving for the uptake of ACPC, glycine, L-methionine and L-proline, the existance of a new site was postulated to account for the entry of AIB into mouse pancreas.

(6) Although the tissue steady-state level of GABA was extremely low, mediated entry was still involved. GABA was an inhibitor of the uptake of the neutral amino acids L-methionine, glycine, AIB and ACPC.

(7) The uptake of L-lysine was greatly affected by pH, while neutral amino acid uptake was relatively insensitive to pH.

(8) Taurine at a concentration of 60 mM caused reduction in the steady-state uptakes of ACPC and glycine, but not of L-lysine. GABA

at a concentration of 60 mM inhibited glycine and L-methionine steady-state uptake, but not that of L-lysine.

(9) DNP, at a concentration of 0.1 mM, caused approximately the same percentage decrease in the uptake of L-lysine, L-tryptophan, AIB, L-methionine, glycine, L-proline and ACPC.

(10) In addition to major suppression of neutral amino acid uptake by a sufficient level of other neutral amino acids, there was also a large reduction in basic amino acid uptake by a sufficient level of several neutral amino acids. A sufficient concentration of L-lysine (basic) inhibited the uptake of glycine and L-methionine to a small extent and the uptake of ACPC to a much greater extent.

(11) The tissue steady-state accumulation levels were (in decreasing order): glycine, ACPC, AIB, L-proline, L-tryptophan, L-methionine, L-lysine, GABA.

(12) NH_4^+, Rb^+, K^+ or Li^+ could not replace external Na^+ in the transport process, whereas Rb^+ could function as a suitable replacement for K^+.

(13) A decrease in either the external Na^+ or K^+ concentration resulted in an increase in the apparent affinity constant, but no change in the maximum concentration gradient.

(14) Kinetic analysis suggested a 1:1 relationship between Na^+ ions and ACPC molecules transported in mouse pancreas.

(15) AIB, ACPC, L-methionine, glycine, L-proline and L-lysine

maintained concentration gradients in the complete absence of external Na^+.

(16) In low Na^+, low K^+ or Na^+-free media, the uptakes of ACPC, glycine, AIB and L-methionine were decreased to approximately the same extent.

(17) In the complete absence of Na^+, amino acid uptake was inhibited by DNP and other amino acids, but not by ouabain.

(18) Exchange diffusion of amino acids in mouse pancreas did not show a dependency on the external Na^+ concentration.

(19) The relative ability of intracellular amino acids to exchange were (in decreasing order): L-methionine, ACPC, L-tryptophan, glycine, AIB, L-proline, L-lysine. When present extracellularly, the short-chain neutral amino acids (glycine, AIB, proline, etc.) exchanged very poorly in all instances, while the long-chain neutral amino acids were generally excellent exchangers.

(20) The exchange system could be saturated only at extremely high levels of amino acid inside the cell. The external amino acid was able to saturate the exchange system at quite low concentrations. In the latter case, the affinity constant of the exchange reaction was of the same order as the affinity constant characterizing the transport of the amino acid into mouse pancreas.

(21) The presence of two amino acids (each below the saturation level) in the incubation medium produced a greater stimulation of

efflux of prepacked amino acid than either one present alone. Such a significant increase would be seen if there was competition for the one site during exchange diffusion.

(22) AIB, a significant inhibitor of the transport of ACPC, did not inhibit the homo-exchange diffusion of ACPC.

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