9629

NATIONAL LIBRARY
OTTAWA

BIBLIOTHÈQUE NATIONALE
OTTAWA

NAME OF AUTHOR... F. H. Osman.....

TITLE OF THESIS.. L-amino-iso-butyric acid uptake in rabbit duodenum mucosa....

.....

UNIVERSITY..... of Alberta.....

DEGREE FOR WHICH THESIS WAS PRESENTED.... Ph.D.....

YEAR THIS DEGREE GRANTED.....1971.....

Permission is hereby granted to THE NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(Signed)... F. H. Osman

PERMANENT ADDRESS:

..Dept. Pharmacology
...Assiut University
.....Assiut, Egypt

DATED.. 14. Sept. 1971

NL-91 (10-68)

THE UNIVERSITY OF ALBERTA

α-AMINOISOBUTYRIC ACID UPTAKE BY RABBIT DETRUSOR MUSCLE

by

(C) FIKRY HASSAN OSMAN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE SUTDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

FALL, 1971

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "α-Aminoisobutyric Acid Uptake by Rabbit Detrusor Muscle" submitted by Fikry Hassan Osman in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

.....
Supervisor

.....

.....

.....

.....

.....

.....
External Examiner

Date..September..15..1971....

ABSTRACT

In view of the paucity of information available on the transport of amino acids in isolated smooth muscles, this study was designed to characterise the mechanism of transport of the model amino acid, α-aminoisobutyric acid, in isolated detrusor muscle obtained from male rabbits.

In order to calculate the intracellular content of the amino acid, the volume of the extracellular space had to be determined. For this purpose the influx and efflux of tracer quantities of [^{14}C]-labelled mannitol, sucrose, inulin and dextran were studied. These compounds were used because they have widely varying molecular weights. The influx of mannitol and sucrose reached equilibration after 1 - 2 hours at a volume of about 60 ml/100gm while inulin and light-dextran reached equilibration after about 2 hours at a volume of about 43 ml/100 gm. The uptake of heavy-dextran was extremely slow, a space of 43 ml/100 gm being occupied only after 4 hours. The efflux of mannitol, sucrose and inulin was incomplete after 6 hours and was multicompartmental in nature; the time course of efflux could be described by the sum of three exponentials plus a 'bound' fraction. The size of the fast fraction of efflux of mannitol and sucrose was approximately the same as that of the total volume of distribution of inulin. Following severe metabolic inhibition, the volumes of distribution of sucrose and mannitol approached the total tissue water while those of inulin and dextran were not altered significantly. The large volumes of distribution of mannitol and sucrose may reflect intracellular penetration. Of the markers

studied, inulin appeared to be the marker of choice for the measurement of the extracellular space in this tissue.

α-aminoisobutyric acid was accumulated slowly against the concentration gradient, a steady state being reached after prolonged periods of incubation. Uptake was found to consist of two components: a saturable process with an apparent "Km" of 0.73 mM and "Vmax" of 1.8 μmoles/ml intracellular H_2O/hour and a non saturable process (within the concentrations used) with a "Km" of 310 mM and "Vmax" of 286 μmoles/ml intracellular H_2O/10 min. The saturable process of uptake was (a) temperature sensitive with Q_{10} of about 2.1 and energy of activation of 13.1 Kcal/mole; (b) energy dependent, being inhibited by 1mM dinitrophenol but not by 1mM iodoacetic acid; (c) inhibited by ouabain; and, (d) dependent on external cations, Na^+ and K^+ being essential for uptake and Mg^{2+} to a lesser extent. Na^+ could not be substituted for by other cations and altered the apparent "Vmax" of uptake but did not change the "Km". The amino acid fluxes did not appear to be significantly affected by the magnitude of the Na^+-gradient across the cell membrane. Uptake was not significantly altered by exposure to iodoacetic acid even though the Na^+ pump was inhibited and the Na^+-gradient reduced. It is possible that the energy for transport of the amino acid in this tissue is not determined by the Na^+-gradient alone but possibly rather from hydrolysis of ATP by the membrane Na^+/K^+-activated ATPase.

The structural requirements for the transport site of α-aminoisobutyric acid were studied by determining the effect of various compounds on the uptake of this amino acid. The transport system

exhibited a marked preference for the L-isomer. The possession and nature of a side chain attached to the α-carbon were important determinants of affinity. Both a carboxyl and an amino group were essential for affinity although some increase in the size of the latter group was still compatible with affinity for the transport site. It also appeared that these groups had to be attached to the α-carbon atom since β- and γ-amino acids had no apparent affinity for the transport site.

ACKNOWLEDGEMENT

It has now become a stereotyped convention to open such academic works with a series of acknowledgements, the phraseology and import of which suggest inevitably tedium and inoriginality.

With this necessary apology in mind, I should like, however, to express my deepest gratitude to my supervisor, Dr. D. M. Paton, who guided my research since its inception, carefully read the manuscript, and contributed valuable criticisms and suggestions, all being inspired by the subtle mixture of his exceptional scholarship and good humour.

I equally acknowledge, with a genuine pleasure, the kindnesses of all my supervisory committee, who spared no effort to facilitate my research throughout its phases, and accorded me all pertinent assistance.

Both to the University of Alberta and the Canadian Heart Foundation, I owe a debt of gratitude; for had it not been for the GTA and the Fellowship they had granted me, I would hardly ever been able to prepare for this dissertation, let alone to bring it near fruition.

My indebtedness to my home country is beyond any verbal recognition: I am inordinately grateful to my Alma Mater, the Assiut University, for having allowed me a paid study leave, and to the U. A. R. Cultural and Educational Bureau in Washington for having, inter alia, defrayed the cost of preparing and typing this thesis.

If I happen to err in the course of this study, which is only human, I should be alone held responsible for any such consequences.

This investigation was supported by a grant from the Medical Research Council of Canada to Dr. D.M. Paton.

TO MY MOTHER AND FATHER

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES - continued.

LIST OF FIGURES

LIST OF FIGURES - continued.

LIST OF FIGURES - continued. Page

I. INTRODUCTION

I. INTRODUCTION

It is now generally accepted that amino acids serve as precursors for protein synthesis (Simkin, 1959). One of the characteristics of an actively metabolizing cell is its capacity to concentrate amino acids intracellularly in excess of their concentration in the extracellular medium (Van Slyke and Meyer, 1913; Luck, 1928; Christensen, 1959). The role of this large intracellular amino acid pool in cellular metabolism has not been defined but has been assumed to be an obligatory precursor pool for protein synthesis. This assumption has been seriously questioned and the results of Kipnis et al., (1961) indicated that the intracellular amino acid pool is functionally heterogenous (compartmentalized) with respect to protein synthesis i.e. it is not an obligatory intermediate in protein anabolism. In studying the functional significance of amino acid transport in protein synthesis, the mechanism of amino acid transport is an initial step in defining this relationship.

The transport of amino acids into various tissue cells was studied as early as 1913 when Van Slyke and Meyer studied the distribution of intravenously injected solutions of various amino acids in a wide variety of tissues. They showed that the free amino acids in the tissues were present in much higher concentration than in plasma and that amino acids entered the tissues against their concentration gradient. Following this, extensive work has been done under in vivo conditions (Chase and Lewis, 1934; Bolton and Wright, 1937; Hober and Hober, 1937; Beyer et al., 1947; Clark et al., 1951). However, an axiom of biochemical advance is that a phenomenon should be studied in a simple form, if possible in an isolated form, in order to under-

stand it. It was not until 1949 that the first study of amino acid uptake under *in vitro* conditions was done by Stern *et al*. These workers examined the uptake of glutamate by brain cortex slices and investigated the role played by metabolic events and energetics of brain cells for the concentrative assimilation of amino acids. In the same year, Christensen and Streicher (1949) and Christensen *et al*., (1949) made an extensive study of the transport of glycine into isolated diaphragm and examined the effects of various agents on the maintenance and inhibition of its concentrating activity. The use of intestinal preparations for studying the transfer of amino acids across the intestinal wall was first done by Wiseman (1951). He was able to show that the properties of this organ *in vivo* could be largely duplicated *in vitro* and that specialized mechanisms for amino acid transport were present in the intestine.

The first animal cells to be used in the form of cell suspensions to study amino acid transport were duck and human erythrocytes (Christensen *et al*., 1952c),rabbit reticulocytes (Riggs *et al*., 1952) and Ehrlich mouse ascites tumour cells (Christensen and Riggs, 1952). Subsequently interest in this field of transport has rapidly accumulated and many studies on amino acid transport have since been undertaken. These studies have been performed in a wide variety of tissue preparations including kidney cortex (Rosenberg *et al*. 1961), pancreas (Begin and Scholefield, 1963), fat cells (Herra and Renold, 1960), bone cells (Finerman and Rosenberg, 1966), liver cells (Crawhall and Segal, 1968), uterus (Riggs *et al*. 1968), ocular fluids and lens (Reddy and Kinsey, 1962), ovaries (Rubinstein and Ahren, 1969), heart (Scharff and Wool,

1965) and peripheral nerves (Caston and Singer, 1969). Studies on the transport of amino acids in smooth muscles have been less frequent. The transport of amino acids into estrogen-primed uterine horns has been studied (Noall and Allen, 1961; Roskoski and Steiner, 1967a, 1967b; Griffin and Szego, 1968; Riggs et al., 1968) but the transport into the myometrium alone was not characterized in these studies since the endometrium was not removed. Similar studies have also been performed using everted intestinal segments; in these studies the transport from the mucosal to the serosal surfaces was examined without discriminating between mucosal transport and the transport through the muscle layer (Wilson and Wiseman, 1954; Cohen and Huang, 1964; Rosenberg et al., 1965; Munck, 1968 a).

The aim of the present study was to investigate the characteristics of transport of α-aminoisobutyric acid (AIB) in the isolated rabbit detrusor muscle preparation. AIB was chosen for the study since it is non-metabolizable (Cammarata and Cohen, 1950; Lang and Oster, 1953) and its intracellular content represents the amount which is being transported through the cell membrane. In addition, AIB has been found in other tissues to be transported in a similar way to that of some naturally occurring amino acids (Noall et al., 1957; Akedo and Christensen, 1962 b).

The questions to which answers were sought were:

1) What is the volume of the extracellular space in detrusor muscle ? Since the calculation of the intracellular content of an amino acid depends on the amount present in the extracellular

space, an estimate of the volume of extracellular space had to be known.

2) Does the uptake of AIB change with time; in what direction does it change if at all ? Is it a diffusion process or an uphill transport phenomenon ?

3) What is the effect of increasing the external AIB concentration on its uptake by the muscle tissue ? Does it increase linearly or is it saturable at a certain concentration ? If it is saturable does the data obtained fit with the Michaelis-Menten kinetics for enzyme action ? What are the values of the kinetic parameters, K_m and V_{max} so obtained ?

4) Is the uptake of the amino acid temperature dependent ? If it is, what is the temperature coefficient (Q_{10}) and the energy of activation of the reaction ?

5) What are the effects of various metabolic inhibitors on the uptake ?

6) Do changes in the ionic composition of the incubating medium effect the transport of AIB through the cell membrane ? If the transport is ion dependent, what is the principal ion(s) that effects the uptake process ?

7) If the amino acid is transported against its concentration gradient, is the energy utilized linked directly to hydrolysis of ATP or to a coupled movement of another substance down its electro chemical gradient ?

8) What is the effect of other naturally occurring amino acids on

the transport of AIB ? For example, do other amino acids compete for the same transport system ? If so, what is the structural specificity for the system required for AIB transport ?

II. LITERATURE REVIEW

II. LITERATURE REVIEW

A. EXTRACELLULAR SPACE

Calculation of the uptake and distribution of any substance across the cell membrane requires prior measurement of extracellular space. It is well known that any tissue may be divided morphologically into two different phases, namely the intracellular and extracellular phases. The intracellular phase is considered to consist of those cells which perform the specific function of the tissue (e.g., for muscle, the muscle cells). The extracellular space is a complex structure, (Day, 1952; Meyer et al., 1956; Haust, 1965); assigned to it are certain fluids and the supporting connective tissue structures which surround the cells. Certain cells are also usually included in the extracellular space, i.e., fibroblasts in the connective tissue, and cells of the blood vessels (Danielson, 1964).

A precise measure of the percentage of tissue fluid which is extracellular is essential if one is to obtain meaningful values for the concentrations of solutes or water within the cell. Particularly in cases of solutes whose intracellular content is low, the correct estimation of the extracellular space is of utmost importance since an erroneous value for the extracellular space may lend to the conclusion that these solutes are altogether absent in the cell while in fact they are present in sizable concentrations (or perhaps vice versa). Schultz et al. (1966) showed that underestimating the value of the true extracellular space lowers the intracellular concentration of all substances concentrated by the cell and increases the intracellular concen-

tration of all substances whose concentration in the cell is lower than that in the surrounding medium. The measurement of the extracellular space could be achieved by more than one method (Sandow, 1939; Conway, 1957; Prosser et al., 1960); one of these is by utilizing the electron microscope. Calculations of extracellular space within bundles have been made from many measurements for both transverse and longitudinal sections. The extracellular areas on the micrograph were determined by placing a grid over low power micrographs and counting squares (Prosser et al., 1960; Lane and Rhodin, 1964), by cutting pieces of tracing paper along the cell boundaries and weighing the pieces (Villamil et al., 1968; Yamanchi and Burnstock, 1969) or by the use of a planimeter (Goodford and Hermansen, 1961; Villamil et al. 1968).

The most commonly used method for the estimation of the extracellular fluid volume is based on the distribution of a probe molecule within this compartment. An implicit assumption is that within the extracellular compartment, there exists the same concentration of probe molecule as in the fluid surrounding the tissue. An ideal extracellular marker (see Danielson, 1964) should penetrate the extracellular space without penetrating the cells and be homogenously distributed in the extracellular space without being adsorbed or fixed to any extracellular site. In addition, it should not be broken down or metabolized. Although a large number of substances of different chemical composition, electrical charge, and molecular weight, have been used for the determination of the extracellular space, there is no concensus on the identity of substance or substances which measure the

extracellular space of all tissues and only this space.

Thus determination of the extracellular space is not without problems. From Table I it can be seen how the values for the extracellular space in the same muscle vary according to the method used for its determination.

Histological methods usually give lower values than those calculated from the uptake of various markers. This may be due to shrinkage of the tissue during fixation (Burnstock et al., 1963) or due to errors in the measurements because the surface of smooth muscle cells shows many indentations or protuberances and there are narrow and tortuous passages between the cells (Caesar et al. 1957). Another contributing factor is that for electron microscopic examination, the areas of the preparation selected for measurements are these which consist mainly of functional muscle bundles (Burnstock, 1970).

Two groups of substances have been used in attemps at the measurement of the extracellular space: (1) Electrolytes such as Na, Cl. thiocyanates, bromides, iodides, thiosulfates, etc... (2) Non electrolytes as inulin, mannitol, sucrose, dextran, raffinose, etc... The difference between the above markers lies in their ionic charge and/or their molecular size. As far as the ionic charge is concerned, Goodford and Hermansen (1961) found that the extracellular space in guinea pig taenia coli measured with inulin and polyglucose was less than that measured by the rapid phase of efflux of Na^+. However, ethanesulfonate (Goodford and Lullmann, 1962), a larger ion, gave a space similar to that measured with the electrically neutral

TABLE I

VARIOUS ESTIMATES OF THE EXTRACELLULAR SPACE IN GUINEA PIG TAENIA COLI.

Method	Extracellular space %	References
Electron microscopy	12	Prosser *et al.* (1960)
Electron microscopy	20	Goodford and Hermansen, (1961)
Polyglucose	22	Goodford and Hermansen, (1961)
Inulin	30	Born, (1962)
Ethanesulfonate	33.8	Casteels, (1966)
Sorbitol	40	Goodford and Leach, (1966)
Co. EDTA	44	Brading and Jones, (1969)
Sucrose	47	Durbin and Monson, (1961)
Sodium	48.8	Daniel, (1958)
Cobalticyanide	50	Goodford, (1967)
Chloride	59.6	Daniel, (1958)

polysaccharide, sorbitol (Goodford and Leach, 1964). The large ionic spaces, therefore, are not due to their ionic charge. Ogston and Phelps (1961) found that large molecules can be excluded from some areas of the extracellular space by the mucopolysaccharide, hyaluronic acid. To extend this observation, the relationship between the molecular size and the measured extracellular space has been determined. (Barr and Malvin, 1965; Goodford and Leach, 1966). The latter authors observed a negative linear correlation between the molecular size and measured volume of the extracellular space, i.e., the measured volume was large when small ions or molecules were used for the determination and small when large molecules were used even with identical experimental procedures.

On measuring the inulin space in tissues pretreated with hyaluronidase (Goodford and Leach, 1966), the volume of distribution of inulin was increased although the wet weight and ionic composition of tissues were unchanged. This supports the suggestion made by Ogston and Phelps (1961) that hyaluronic acid prevents the free entry of large molecules such as inulin into the extracellular space by steric hinderance.

On the other hand, Villamil et al. (1968) using the isolated carotid artery of the dog obtained an anatomically-determined space which exceeded that measured by analytical methods. This might be expected if one considers that these extracellular markers are excluded from some areas of extracellular space or that the anatomical space contains large and unmeasured amounts of solids. Treatment of the tissues with hyaluronidase did not, however, have any significant ef-

fect on inulin space although histological staining sutdies showed that hyaluronidase affected the hyaluronic acid in the extracellular compartment. Since Day (1950) observed that the effect of hyaluronidase was counteracted by dilute solutions of certain substances of large molecular size, it would be difficult to explain why this happens in the carotid artery of the dog but not in the guinea pig taenia coli.

Although the large volumes of distribution of substances of small molecular size was taken as an indication of intracellular penetration (Bozler and Lavine, 1958), an ingenious method was adopted by Page (1962) using an intracellular microelectrode technique to determine whether a molecule enters cell at an appreciable rate or whether it is effectively excluded from the cytoplasm by the cell membrane. The principle of this method referred to as the osmotic gradient method (Fig. 1) is to preincubate the tissue in hypotonic high K^+ solution in which the permeant Cl^- is replaced by the impermeant SO_4^{2-}, thereby causing the cell to lose its diffusible Cl^-. This design makes the membrane behave approximately like a K^+ electrode. The tissue is then transferred to an isotonic solution in which the osmotic deficit is made up by the test molecule. The increase in external osmolarity causes the cell to lose water and this results in a rise of the intracellular K^+ concentration. The increase in the ratio of internal to external K^+ causes an increase in the membrane potential. The persistent elevation of the membrane potential denotes a non-permeating solute while a fall in the membrane potential after the initial rise

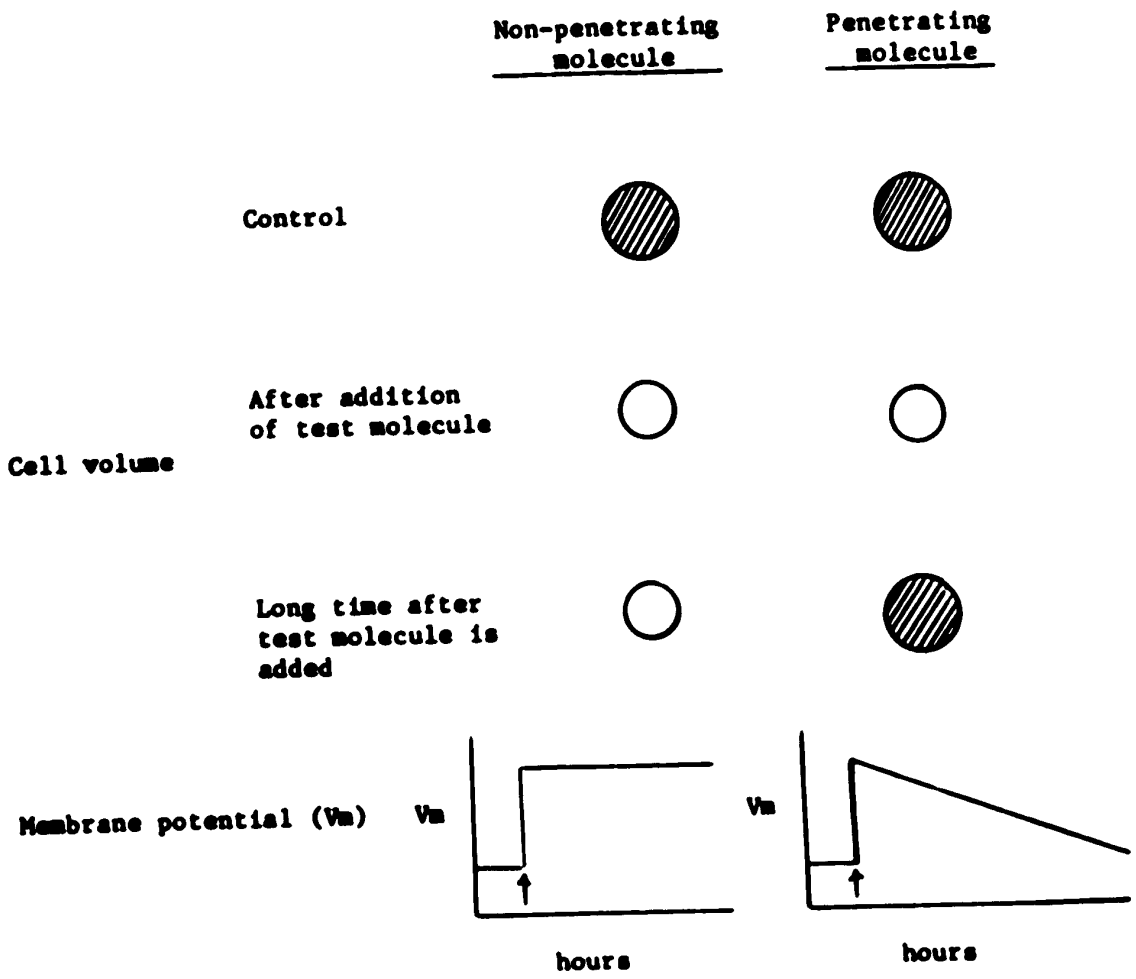


Figure 1. Osmotic Gradient Method.

denotes that the test molecules enters into the cells.

The results of this method led to an analysis of the simultaneous uptake of inulin and mannitol by the extracellular space of quiescent cat papillary muscle. This analysis indicated that both inulin and mannitol are non-penetrating molecules and since there was a large difference in the volumes of distribution of inulin and mannitol, the conclusion is that the extracellular space is not a homogeneous compartment.

Recent work using autoradiographic techniques has shown that extracellular markers of different molecular sizes can gain access into nerve cells (Nicholls and Wolfe, 1967; Brown et al., 1969) although the amount detected intracellularly was very small.

Faced with the problem of the lack of homogeneity of the extracellular space, Barr and Malvin (1965) used values obtained using various markers to calculate the intracellular content of Na^+ and K^+ in circular muscle of the dog jejunum. Any marker that gives a space larger than the Na^+ space cannot represent the true extracellular space but is in fact an overestimate. However, calculations of this sort lead to misinterpretations since it has been shown that Na^+ is bound to the acid mucopolysaccharide-protein component of the paracellular matrix (Palaty et al., 1969).

In contrast to the above observations, Weiss (1966) used the longitudinal muscle of the guinea pig ileum and did not find any significant differences in the volumes of distribution of sucrose, inulin, or mannitol. His washout experiments showed that the efflux of inulin

is slower than that of mannitol and sucrose. Also, the curvature in the semilog plot indicates that the efflux of these markers is not obeying a single exponential function but is complex in nature.

While the use of different markers gave different volumes of distribution, using the same marker but with different labels also gave different values. Dryden and Manery(1970) found that the tritiated inulin space in the thigh muscles of the frog was higher than that measured by [^{14}C]-inulin in the same muscle.

In retrospect to the first attempt to determine by Hermann in 1888 the extracellular space (see Fenn, 1936 for references), there appears to be no single method to date which can give precise measurement of the true extracellular space.

B. AMINO ACID TRANSPORT

1. Time Course of Uptake

The transport system of any given amino acid differs from one tissue to another. Using the same concentrations of α-aminoisobutyric acid in the incubation medium, it was found that steady state levels were reached after about 90 minutes in toad bladder (Thier, 1968) and 4 hours in foetal rat calverium (Finerman and Rosenberg, 1966), whereas in rat liver slices, the uptake was linear for up to 3 hours with no tendency to saturation (Crawhall and Segal, 1968).

The uptake of glycine by ascites tumour cell, was extremely rapid with half maximal uptake being attained in about 2 minutes irrespective of whether the concentration of glycine in the external medium was 0.5 or 5 mM (Heinz, 1954). Subsequent work by Johnstone and Scholefield (1959) confirmed the rapid uptake of glycine but these workers found that about 7 minutes incubation was required to achieve half maximal uptake. However later work by Oxander and Christensen (1963 a, 1963 b) showed that the uptake of glycine by these tumour cells was relatively slow and 50% of the steady state levels was reached after about 20 minutes. It seems therefore that different experimental conditions affect the rate of uptake of an amino acid by the same kind of cells.

The steady state levels attained by amino acids do not necessarily bear any relationship to their initial rates of uptake. For example in mouse pancreas although the initial rate of uptake of 1- amino 2- methylcyclopentanecarboxylic acid was lower than that of valine and

ethionine, yet its distribution ratio after 2 hours was higher than these two amino acids (Begin and Scholefield, 1965). Similarly in Ehrlich cells both proline and glycine attained a similar distribution ratio after 30 minutes although the initial rate of glycine transport was about one half that of proline (Christensen et al. 1962). In addition the initial rate of methionine uptake was 3 times as that of α - aminoisobutyric acid although the final distribution ratio reached after half an hour for methionine was half that observed with α-aminoisobutyric acid (Christensen et al. 1962).

2. Effects of Substrate Concentration

The distribution ratio of amino acids between the intra - and extracellular water depends not only on the nature of the amino acid and incubation period but also on its concentration in the external medium. The higher the external concentration, the lower is the distribution ratio (Paine and Heinz, 1960). In 1952 Christensen and Riggs showed that the concentrative uptake of glycine by Ehrlich ascites cells was not a linear function of its external concentration, but rather was curvilinear in nature. Subsequent work by Heinz (1954) led to kinetic analysis of glycine transport in these cells and the influx of glycine could be described in terms of the Michaelis Menten equation for enzyme reactions. The value of K_m for glycine at 37° was found to be 3 to 7 mM. Tenenhouse and Quastel (1960) have confirmed these findings but instead of using the initial velocity in their analysis, they used the steady state level attained by the cells. In addition they have reported that with the amino acid analog, S-ethyl cysteine,

the double reciprocal plot for concentrations below 8 mM was linear whereas above this concentration, the plot curved towards the origin. They suggested that the transport of S-ethyl cysteine at high concentrations did not obey saturation kinetics. This behaviour has been subsequently observed in many tissues where in addition to the usual saturable component at low external concentrations, there is another component which fails to saturate at reasonable concentrations of the amino acid (Akedo and Christensen, 1962 a; Rosenberg et al., 1961; Vidaver, 1964; Riggs et al., 1968; Scriver and Mohyuddin, 1968; Touabi and Jeanrenaud, 1969). Akedo and Christensen (1962 b) have measured this last component by determining the uptake at higher concentrations and then extrapolating it to infinite concentration. If this non-saturable component was not subtracted from the total entry rate of the amino acid, the double reciprocal plot curved downwards towards the origin at high solute concentrations. It was assumed that this non-saturable uptake occurred by diffusion or by a mediated transport which failed to be saturated within the concentration range used (Akedo and Christensen, 1962 b). If it were practical to test solute levels at much higher concentrations, saturability might become evident. However examination of this non-saturable uptake of amino acids into Ehrlich cells and into rat jejunum showed that this component exhibited chemical specificity, pH dependence and temperature sensitivity (Christensen and Liang, 1966). This indicated that it was a chemically mediated transport and did not occur by simple diffusion.

3. Temperature Dependence

The transport of amino acids in various tissue cells has been shown to be sensitive to changes in temperature; an increase in the rate of transport and in the steady state levels were obtained on elevating the temperature of the incubation medium (Riggs et al., 1954; Rosenberg et al., 1961; Chirigos et al., 1962; Helmreich and Kipnis, 1962; Oxander and Christensen, 1963b; Kipnis and Parrish, 1965; Eavenson and Christensen, 1967; Rosenbusch et al., 1967; Nelson and Lerner, 1970). Moreover, the optimum temperature for maximal uptake of amino acids sometimes varies with the amino acid being transported. In tumour cells for example, whereas glycine uptake reached its peak at about 37°C, the maximal uptake of S-ethylcysteine was achieved at 25°C (Tenenhouse and Quastel, 1960). However, in avian small intestine the maximum velocity for methionine uptake was increased as the temperature was elevated to 52°C (Nelson and Lerner, 1970). Using initial velocities rather than steady state levels may be the reason why the latter authors got an elevated uptake values at 52°C. Sterling and Henderson (1963) have shown that the initial rate of uptake of aminocyclopentanecarboxylic acid in ascites tumour cells at 37.5°C was nearly twice that at 26° although the final steady state levels were equal at both temperatures.

In rat kidney cortex slices, cysteine accumulation showed a somewhat paradoxical effects when its dependence on temperature was studied (Segal and Crawhall; 1968). At short incubation periods (10 minutes) the uptake of cysteine was diminished as the temperature

was reduced to 20°C from 37°C with a temperature coefficient of 1.5 and energy of activation of 7.25 Kcal/mole. With further prolongation of the incubation period, the steady state distribution ratios at 20°C were nearly twice as those seen at 37°C. This paradox seemed rather surprising and Segal and Crawhall (1968) have suggested that the reason may be due to inhibition of efflux at low temperatures when the incubation period was prolonged.

In the rabbit ileum Curran et al. (1967) studied the temperature dependence of alanine flux across the mucosal border within the range of 23°C to 37°C. In the presence of 140 mM Na^+, a Q_{10} of 4.5 was obtained, whereas in the absence of Na^+, the Q_{10} was about 3.2. They have pointed out that the presence of any experimental error could markedly affect the calculated Q_{10} at room temperature in the absence of Na^+ due to the small amounts of alanine influx obtained under such conditions.

The temperature coefficient for amino acid transport may vary when different ranges of temperatures are used for its calculation. Tenenhouse and Quastel (1960) have shown that the Q_{10} for glycine uptake by tumour cells between 10 and 20°C was about 1.5 while between 25 to 37°C, it reached a value of 2.4. In a recent study Jacquez et al. (1970) have summarized some of the Q_{10}'s and energies of activation for various aspects of amino acid transport in various tissue cells. In addition they investigated in detail the effects of temperature on the kinetic parameters of amino acid transport in Ehrlich ascites cells. The results of their study showed that the maximal flux was reduced with a decrease in temperature whereas the changes in the K_m depended on the

amino acid being transported. As the temperature fell, the K_m for α- aminoisobutyric acid was increased, the K_m for phenylalanine and tryptophan was decreased while that for methionine, glycine and alanine was independent of temperature. The energies of activation for these amino acids fell in the range of 11 to 20 Kcal/mole (Jacquez et al., 1970).

4. Role of Energy

a) Effects of exogenous substrates

Studies performed in tumour cells (solid or ascitic forms) have shown little or no requirement for an exogenous substrate to support maximum uptake of amino acids (cf. Johnstone and Scholefield, 1965). The same was also true for the mouse pancreas where the addition of potential substrates to the incubation medium produced little or no effect on the uptake of glycine or ACPC (α -aminocyclopentanecarboxylic acid) by the pancreatic tissue (Begin and Scholefield, 1964) as it was for the rat uterus where the addition of glucose to the incubation medium did not affect α-aminoisobutyric acid uptake during the 8 hours incubation period (Riggs et al., 1968). This is in direct contrast to the brain cortex where there appears to be an absolute requirement for an exogenous substrate to support maximal uptake of amino acids (Abadom and Scholefield, 1962 a; Gurroff and Udenfriend, 1962; Tsukada et al., 1963). When glucose or other hexoses were added to the external medium, the uptake of tyrosine by rat brain slices was markedly stimulated. Pyruvate also enhanced the uptake but other aerobic substrates were completely ineffective (Gurroff and Udenfriend, 1962). The effects of exogenous substrates were found to be stimulatory to

the concentrating mechanisms because p-hydroxyphenylacetic acid [whose uptake was not concentrative] was not affected by the presence of glucose in the buffer. A similar study was performed by Abadom and Scholefield (1962 b) and they have shown that the ability of various substrates to support amino acid transport in the brain cortex slices was directly proportional to the amount of ATP generated. In contrast they have shown that some aerobic substrates were able to support glycine transport but with less efficiency than the hexoses.

The ability of glucose to influence the concentrative uptake of amino acids was also examined in intestinal preparations. Glucose unlike other actively transported sugars was found to enhance the transport of amino acids in the small intestine of the rat (Newey and Smyth, 1964 b; Munck, 1968 a). The stimulating effect of glucose on transport of amino acids was attributed to its ability to provide additional energy by increasing the availability of ATP to support the uphill transport (Munck, 1968 a; Newey and Smyth, 1964 b). Furthermore, in the presence of glucose, the inhibitory effects of galactose on the transport of proline and valine by the rat small intestine was completely eliminated as the energy supply would be increased in the presence of glucose (Munck, 1968 a). Although in rat and rabbit small intestine there is evidence against interactions between sugars and amino acids at the carrier level (Chez et al., 1966; Munck, 1967, 1968 b), in the hamster small intestine it was proposed that both sugars and amino acids share a common carrier (Alvarado, 1966; Hindmarsh et al. 1966).

b. Effects of Anaerobiasis and Metabolic Inhibitors

It is well known that in downhill transport, the driving force is the concentration difference between the two sides of the membrane. In uphill transport, the free energy of the substrate increases at the expense of the metabolic energy produced by the cell. The first approach to the problem of identifying the route by which energy from metabolism is supplied by active transport mechanisms is to examine the action of metabolic inhibitors on the system.

Christensen and Riggs (1952) found that the uptake of glycine by Ehrlich ascites cells was markedly reduced under anaerobic conditions and in absence of glucose compared with that observed under aerobic conditions. In the presence of glucose the uptake was found to be the same whether the incubations were performed under aerobic or anaerobic conditions (Johnstone, 1959). Incubation of cells with DNP under anaerobic conditions did not affect the uptake of glycine as long as glucose was present in the incubation medium (Tenenhouse and Quastel 1960). In addition inhibition of the uptake was produced by IAA under anaerobic but not under aerobic conditions. This led the latter workers to suggest that the energy from glycolysis or from respiration could serve for the concentrative uptake of amino acids by these cells. These energy sources have also been postulated for amino acid uptake in the small intestine (Baker and Copp, 1965; Nelson and Lerner, 1970), uterus (Riggs et al. 1968), liver (Crawhall and Segal, 1968; Tews and Harper, 1969) and skeletal muscle (Bombara and Bergamini, 1968).

The accumulation of amino acids by kidney seemed to be dependent

solely on aerobic metabolism. Rosenberg et al. (1961) have shown that the accumulation of glycine, histidine, phenylalanine and α-amino-isobutyric acid by renal cortex slices was completely abolished under anaerobic conditions. Similarly, the concentrative accumulation of glycine in brain cortex slices was virtually abolished in absence of oxygen atmosphere (Abadom and Scholefield, 1962 a). The latter authors reported also that the uptake of glycine in presence of DNP and glucose was markedly inhibited and this inhibitory effect was accompanied by a concomitant reduction in ATP levels. This is in contrast to tyrosine whose transport was not affected by DNP so long as glucose was present in the external medium (Gurroff et al., 1961).

In red blood cells Christensen et al. (1952 c) have observed remarkable insensitivity of the concentrative uptake of glycine and alanine by duck and human erythrocytes to anaerobiasis and various metabolic inhibitors. However in pigeon erythrocytes these agents have a pronounced inhibitory effects (Eavenson and Christensen, 1967) on the uptake of these two amino acids.

The effects of metabolic inhibitors on the unidirectional fluxes of alanine across the mucosal border of the rabbit ileum was studied by Chez et al. (1967) and they have reported that the decrease in net uptake was mainly due to an increased efflux across the mucosal border although a slight decrease in influx was also observed.

Experimental evidence has shown that the transport reactions are less sensitive to the action of uncouplers and respiratory inhibitors than are other energy dependent processes. Johnstone and

Scholefield (1959) have reported that DNP in concentration of 0.05 mM which completely inhibited the incorporation of glycine into proteins of Ehrlich ascites tumour cells, had considerably less effect on the transport of glycine into the cells. The same finding was also observed in tumour slices where the same concentration of DNP was without effect on amino acid transport but inhibited the labeling of proteins by more than 70%(Ellis and Scholefield, 1961). Furthermore in pancreatic tissues a concentration of 0.04 mM DNP produced a decrease of approximately 80% of the incorporation of glycine into the proteins, whereas a concentration of 0.08mM was required to decrease the transport by 50% (Begin and Scholefield, 1964).

5. Ionic Requirement for Transport

The implication of intracellular and extracellular Na^+ and K^+ in the cellular accumulation of amino acids has been studied by various investigators. Early studies on the role of these monovalent cations in amino acid transport suggested that K^+ played a more important role than Na^+. In some of their early studies, Christensen et al. (1952 c) found that replacement of Na^+ by K^+ in the incubation medium led to a reduction in the level to which glycine and alanine were accumulated by duck erythrocytes. Similar studies performed in Ehrlich ascites tumour cells showed that a net efflux of K^+ and partial replacement of intracellular K^+ by Na^+ accompanied the uptake of the unnatural amino acid α, γ,diaminobutyric acid by these cells; the concentrative uptake was inhibited by replacement of extracellular Na^+ by K^+ or choline (Christensen et al. (1952 b). Subsequent work by Riggs et al. (1958)

suggested that the uptake of amino acids by Ehrlich ascites cells depended on the intracellular K^+ levels. In cells made deficient in K^+ and then placed in normal K^+ containing medium, their ability to accumulate amino acids was not restored until the intracellular K^+ level was increased. The latter workers also pointed out that the efflux of K^+ was accompanied by an influx of Na^+ and suggested that the amino acid transport might depend on the Na-gradient. The choice between K^+ efflux and Na^+ influx as the specific driving influence was decided upon when they found that pyridoxal and indole acetate which stimulated glycine transport caused K^+ loss from the cell accompanied by minimal Na^+ replacement. Thus the hypothesis was advanced that the energy inherent in the asymmetric distribution of K^+ between the intracellular and extracellular medium was the driving force for glycine accumulation. One mechanism suggested was that K^+ efflux drove glycine influx by exchange diffusion (Riggs et al. 1958). However the results of Hempling and Hare (1961) did not corroborate this hypothesis and their data provided two reasons for rejecting the hypothesis that K-efflux drives glycine influx by exchange diffusion. Firstly, the energy available by movement of K^+ down its electrochemical potential gradient was found to be less than the energy required to pump glycine into the tumour cells; therefore, from energetic considerations K^+ efflux could not drive glycine influx. Secondly, at the inner surface of the membrane, the affinity of glycine for a postulated common carrier was 25 times that of K^+. If exchange diffusion with K^+ were to account for glycine uptake, then the affinity of K^+ should be greater than that of glycine

on the inside of the membrane. Kromphardt et al. (1963) found that glycine uptake depended on extracellular Na^+, not intracellular K^+. This was based on their findings that lowering of internal K^+ from 168 mM to 71 mM did not alter glycine transport, while a reduction of the level of external Na^+ was accompanied by a concomitant reduction in the uptake of glycine. Furthermore, the work of Riggs et al. (1958) was reevaluated by Wheeler et al. (1965) who concluded that the results actually gave evidence for a dependence of amino acid flux on Na gradient.

The active transport of amino acid in most tissue cells appeared to require the presence of Na^+. The active transport of D-glutamate in rat cerebral cortex was found to be dependent on Na^+. Deletion of Na^+ on the other hand, resulted in a complete failure of the active transport process (Takagaki et al. 1959). α-aminoisobutyric acid accumulation in human leucocytes (Yunis et al., 1962, 1963), Ehrlich cells (Inui and Christensen, 1966) and intestinal cells (Rosenberg et al. 1965) was similarly dependent on Na^+ as was the transport of serine into adrenal cortex slices (Sharma et al. 1964) and the transport of amino acids into renal cortex slices (Fox et al. 1964).

Although all Na-dependent transport processes of amino acids appear to be capable of uphill transport, the reverse may not be true, i.e., all amino acid concentrative processes may not be Na-dependent. For example, rat calveria were capable of the uphill transport of glycine and α-aminoisobutyric acid in the complete absence of Na^+ (Finerman and Rosenberg, 1966) as was the transport of methionine in

Ehrlich cells (Inui and Christensen, 1966). Also, the accumulation of lysine in kidney cortex slices (Fox et al. 1964) and in isolated rabbit ileum (Munck and Schultz, 1969) was dependent partially on external Na^+ although its concentration by the epithelial cells of toad bladder (Thier, 1968) and Ehrlich ascites tumour cells (Christensen and Liang, 1966) did not require any external Na^+.

In Na^+ dependent transport systems, the requirement for Na^+ seems to be specific. Many substances, electrolytes and non-electrolytes, have been used to replace Na^+ in the external medium but none of them were capable of reversing the inhibition of transport resulting from omission of Na^+ from the external medium (see Schultz and Curran, 1970 for references). However, substitutions for Na^+ frequently produced different effects on the transport process. For example, the uptake of α-amino isobutyric acid by rat liver slices was found to be greater when K^+ or sucrose was used to replace Na^+ than when Li^+ or choline was used as a substituent (Tews and Harper, 1969). Also, the uptake of amino acids by calf thymus nuclei was greater when Li^+ was substituted for Na^+ than when K^+, Cs^+ or Rb^+, was used to replace Na^+ (Allfrey et al. 1961). However, replacement of Na^+ by Li^+, K^+, choline or Tris resulted in the same fall in the rate of alanine uptake by the rabbit reticulocyte irrespective of the cation substituted for Na^+ (Wheeler and Christensen, 1967). The ability of Li^+ to partially substitute for Na^+ has been demonstrated for amino acid transport in several tissues. In isolated thymus nuclei, Na^+ dependent alanine uptake was four times greater in the presence of Li^+ than in

the presence of K^+ (Allfrey et al. 1961). Eddy and Hogg (1969) have shown that glycine influx in Ehrlich ascites tumour cells was stimulated by Li^+. Li^+ was found also to mimic partially the effects of Na^+ in amino acid transport in Ehrlich cells and rabbit reticulocytes (Christensen et al. 1969). Quite recently, Frizzel and Schultz (1970) have shown that Li^+ has a stimulatory effect in the absence of Na^+ on alanine influx across the brush border of the rabbit ileum. In addition, the stimulatory effect of Li^+ was observed in the case of sugar transport in the hamster small intestine. Bihler and Adamic (1967) have shown that when Na^+ was completely replaced by Li^+, sugar equilibration was more rapid than when mannitol, choline, or K^+ were used This Li^+ stimulated effect was inhibited by phlorizin and by other sugars that competed for the same transport system. It was not manifested with non-actively transported sugars nor with the actively transported α-aminoisobutyric acid. Bihler and Adamic suggested that this effect of Li^+ was due to its binding to the sugar carrier with subsequent slight activation of that carrier. However, Goldner et al. (1969) failed to observe any stimulatory effect of Li^+ on 3,O-methylglucose influx across the brush border of the rabbit ileum. That Li^+ could partially mimic the action of Na^+ is very interesting in view of the anionic field strength theory (Eisenman, 1961) which indicates the existence of graded order of selectivities among the alkali metal ions rather than an all-or-none preference for a particular ion in various biological processes.

For maximal transport of amino acids, optimal concentrations of

Na^+ and K^+ are required in the external medium (Takagaki et al., 1959; Yunis et al., 1962; Fox et al., 1964; Rosenberg et al., 1967; Touabi and Jeanrenaud, 1969). Optimal glycine uptake is Ehrlich ascites cells has been observed at approximately 100 mM Na^+ in the external medium (Heinz, 1962) whereas in the brain, a reduction of the Na^+ concentration from 148 to 100 mM has been reported to result in 30% reduction of glycine transport (Abadom and Scholefield, 1962 b). Optimal valine transport has also been seen in the rat intestine when the external Na^+ was 30 mM; below this concentration the uptake was depressed and virtually abolished in the absence of Na^+ (Reiser and Christiansen, 1967).

K^+ in high concentrations had been found to be inhibitory to the transport processes in many tissues (see Schultz and Curran, 1970) and antagonized the action of Na^+ on α-aminoisobutyric acid influx into the intact rat diaphragm (Kipnis and Parrish, 1965), on glycine influx into the mouse ascites tumour cells (Eddy et al., 1967; 1969) and on sugar accumulation by hamster small intestine (Crane et al. 1965). All these authors have suggested that K^+ competes with Na^+ for the same negative site on the carrier. Alternatively, high external K^+ concentrations may produce swelling of the cells which could lead to changes in the functional integrity of the cell membrane and to a non-specific inhibitory effect on the transport processes (Schultz et al., 1966; 1967; Curran et al. 1967)

Lowering of K^+ levels in the external medium impairs the transport process although the effect may not always be apparent. For exam-

ple, whereas the net uptake of tyrosine by rat brain (Gurroff et al., 1961) and of valine by rat intestine (Reiser and Christiansen, 1967) was not affected by omitting K^+ from the external medium, the accumulation of various amino acids in various other tissues was depressed when the tissues were incubated in K^+-free media (Tews and Harper, 1969; Begin and Scholefield, 1964; Touabi and Jeanrenaud, 1969; Fox et al., 1964). The inhibitory effect produced by the omission of K^+ may be secondary to changes in Na^+ extrusion since Na pumping requires an optimal concentration of K^+ in the external medium (Albers, 1967). Under such conditions, the effects of lack of K^+ would mimic the effects of cardiac glycosides in their effects on the transport process. Eddy et al. (1967) found that both ouabain and very low concentrations of K^+ in the external medium lowered the net uptake of glycine by ascites cells while under both conditions the glycine efflux was greatly enhanced.

The requirement for divalent cations has also been examined in a variety of tissues. In the brain, omission of Ca^{2+} inhibited the uptake of α-aminoisobutyrate (Lahiri and Lajtha, 1964), glycine (Abadom and Scholefield, 1962 a), histidine (DeAlmedia et al., 1965) and tyrosine (Gurroff et al., 1961), while those of γ-aminoisobutyric acid, glutamic acid, and glutamine were slightly increased (Tsukada et al., 1963). In liver slices replacement of Ca^{2+} caused a marked inhibition of α-aminoisobutyric acid uptake (Tews and Harper, 1969). The observed effects produced by Ca^{2+} may have been due to changes in ATP levels (Abadom and Scholefield, 1962 a) or to changes in the conformation of the lipoprotein complex of the cell membrane (Manery, 1966). It should be

mentioned, however, that the uptake of valine by the intestine (Reiser and Christiansen, 1967) and α-aminoisobutyric acid by the kidney cortex (Fox et al.1964) were not affected by deletion of Ca^{2+} from the incubation medium.

Mg^{2+} has been found to be stimulatory for amino acid uptake in the brain (Abadom and Scholefield, 1962 a; Guroff et al. 1961), although it has no significant effect on amino acid uptake in kidney (Fox et al. 1964) or liver slices (Tews and Harper, 1969).

6. Effects of Cardiac Glycosides

The cardiac glycosides are known to inhibit the active movements of Na^+ and K^+ across the cell membrane (see Glynn, 1964 for references) by inhibiting the Na^+, K^+-activated ATPase (Skou, 1965). In addition, the active transport of a wide variety of substances in a large number of tissues and in many different species is also depressed by cardiac glycosides (Wolff, 1960; Gonda and Quastel, 1962; Abadom and Scholefield, 1962b; Csaky, 1963; Fox et al., 1964; Parrish and Kipnis, 1964; Berndt and Beechwood, 1965; Csaky and Hara, 1965; Rosenberg et al., 1965; Schultz et al., 1966; Eddy et al., 1967; Field et al.,1967; Newey et al., 1968; Thier, 1968; Hauser, 1969; Tews and Harper, 1969). However, there are also some reports which show that ouabain has no effect on Na-dependent amino acid transport processes. The first observation that ouabain did not inhibit the active transport of amino acids was reported by Paine and Heinz (1960) in their studies in Ehrlich ascites tumour cells. However, this lack of inhibition by ouabain may be due to insufficient exposure time to ouabain since Bittner

and Heinz (1963) observed an appreciable inhibition of glycine transport if the cells were preincubated with ouabain. In the intact rat diaphragm 10^{-4} M ouabain caused a significant inhibition of the active Na^+ transport (intracellular Na^+ increased from 33 mM to 68 mM) without affecting the net uptake of α-aminoisobutyric acid (Kostyo and Schmidt, 1963). In addition, Vidaver (1964) has reported that the Na^+ dependent uptake of glycine by the pigeon red blood cells was not affected by strophanthin and Wheeler and Christensen (1967) have shown that ouabain did not depress the uptake of alanine by the rabbit reticulocytes. The failure of ouabain to inhibit amino acid uptake in such cases may indicate, therefore, that no direct link with active cation transport exists. It should be mentioned that ouabain exerts its effects on the Na^+ dependent uphill transport processes while it does not affect the Na independent concentrative processes. For example, ouabain inhibited lysine transport in the renal cortex slices in presence of Na^+ but the active accumulation of lysine which occurred in absence of Na^+ was unaffected by ouabain (Fox et al. 1964).

Begin and Scholefield (1964) have shown that the uptake of glycine by the isolated pancreas of the mouse was inhibited in the presence of ouabain while the incorporation of that amino acid into proteins was unaffected. This has been held due to the fact that ouabain has no effect on the ATP levels within the cells (Kostyo and Schmidt, 1963). Thus it seems that the action of ouabain is limited only to the transport process.

Concentrations of ouabain required to produce inhibitory effects

on the transport processes of amino acids depend on the tissue used. As is shown in the Table II, the rat kidney requires concentrations of ouabain which are 16 fold greater than those required by the rat bone to produce the same degree of inhibition of glycine transport. Although species differences might be implicated, such trend might be apparent from comparisons of these figures with those from mouse pancreas or Ehrlich ascites tumour cells. Furthermore the response to ouabain also varies within the same tissue depending on the amino acid transported. For example, in slices of renal cortex, ouabain inhibited the uptake of α-aminoisobutyric acid by 60%, glycine by 67%, and lysine by about 50% (Fox et al., 1964). Glycine, with the highest distribution ratio, showed the greatest inhibition by ouabain. The same principle also holds true for bone (Finerman and Rosenberg, 1966). So it seems that the higher the degree of accumulation, the greater is the degree of inhibition by ouabain.

It is well known that hydrolysis of ATP by the membrane Na^+, K^+ activated ATPase furnishes the energy responsible for diverse active processes through the cell membrane. The utilization of this energy is proposed to extrude Na^+ out of the cell and by maintaining the Na^+ concentration gradient across the cell membrane, an uphill transport of the solute can occur through its coupled movement with Na^+ (Crane, 1964). Alternatively, the energy produced from ATP hydrolysis may be used directly to drive the transported solute against its concentration gradient (Csaky, 1963). Thus by preventing ATP hydrolysis the cardiac glycosides can inhibit the uphill movements of amino acids

TABLE II

EFFECTS OF OUABAIN ON GLYCINE TRANSPORT IN SOME TISSUES *

Tissue	Concentration of ouabain (mM)	Inc. Period (min.)	% Inhibition	Reference
Pancreas (mouse)	0.02	60	70	Begin and Scholefield (1964)
Bone (rat)	0.05	30	63	Finerman and Rosenberg (1966)
Ehrlich ascites tumour cells	0.10	60	58	Bittner and Heinz (1963)
Kidney (rat)	0.80	90	67	Fox et al. (1964)

* In calculating the % inhibition, that portion of the uptake which was against the concentration gradient was used.

either directly or indirectly. The distinction between these two alternatives rests on three main lines of evidence (Schultz and Curran, 1970):

1) If ouabain acts directly, then its site of action as well as the site of action of the Na^+-dependent transport process should be the same. However, experiments performed on intestinal preparations have shown that the site of entry of amino acids or sugars into the cell was located at the brush or mucosal border (Schultz and Zalusky, 1964b; Schultz et al., 1967; Stirling and Kinter, 1967; Goldner et al., 1969), while the site of action of ouabain was reported to be located at the serosal border (Schultz and Zalusky, 1964 a; 1964 b: Csaky and Hara, 1965; Field et al., 1967). A direct effect of ouabain, therefore, seems to be unlikely, On the other hand, Kostyo and Schmidt (1963) have shown that in intact rat diaphragm, ouabain inhibited the downhill movement of α-aminoisobutyric acid which might indicate a direct effect of ouabain on the transport mechanism.

2) If ouabain acts indirectly through the increased cellular level of Na^+, then its inhibitory effect on the net uptake should be due to an increased efflux of the solute out of the cell. Eddy et al. (1967) have shown that the efflux of glycine from mouse ascites tumour cells was stimulated by about 60% in presence of 10^{-4} M ouabain when the external Na^+ was 151 mM but not when it was 32 mM. Also the efflux of alanine from the

intestinal cells towards the mucosal solution was greater from ouabain treated tissues (Na^+ loaded) than from low Na^+ tissues (Hajjar et al. 1970). The behaviour of ouabain in this regard, viz, accelerating the efflux of amino acids, was also observed in rat cerebral cortical slices (Gonda and Quastel, 1962). In all these experiments, however the tissues were loaded with the amino acid and the increased intracellular Na^+ concentration facilitated their exit out of the cells. The amino acid appeared to move down while Na^+ was apparently moving up the gradient or vice versa. However, the inhibitory effects of ouabain on amino acid transport in normal physiological medium cannot be ascribed to an increased rate of efflux since under the above conditions both the amino acid and Na gradients are directed inwards.

3) If the inhibitory effect of ouabain is secondary to inhibition of the Na^+ pump with a subsequent loss of the Na^+ gradient across the membrane, then a time period should exist between the inhibition of the Na^+ extrusion mechanism and inhibition of amino acid accumulation (depending on the rate at which Na^+ is accumulated into the cells). Kostyo and Schmidt (1963) have shown that in intact rat diaphragm 10^{-4} M ouabain increased the intracellular level of Na^+ from 33 to 68 mM without any effect on α-aminoisobutyric acid uptake. However, 10^{-3}M strophanthin-K increased the intracellular level of Na^+ from 24 to 55 mEq/L and produced a very marked inhibition of

α- aminoisobutyric acid uptake (Kipnis and Parrish, 1965). It was further demonstrated that higher concentrations of glycosides were needed to inhibit amino acid transport than amounts to produce a marked effect on ion transport. The squill glycosides which were more active on ion transport on a molar basis, were no more active than ouabain on α-aminoisobutyric acid uptake (Kostyo and Schmidt, 1963). This suggests that the actions of the cardiac glycosides on ion transport are dissociated from their actions on amino acid uptake.

Thus the exact mechanism whereby ouabain exerts its inhibitory action on amino acid transport remains unknown due to the lack of concrete evidence supporting one of the aforementioned views.

7. Specificity of the Transport System

a) Optical specificity

In transport studies, comparisons made between enantiomorphic pairs of amino acids showed that differences do exist in various tissues. In hamster intestine Lin et al. (1962) showed that the transport system for neutral amino acids exhibited a preference for the L-stereoisomers, the D-isomers with the exception of methionine not being transported against their concentration gradient. In rat intestine, two forms of carriers were involved in the intestinal transport of neutral amino acids (Newey and Smith, 1964 a). They have been termed methionine and sarcosine carriers (Daniels et al., 1969 b), as methionine was transported mainly by one carrier and sarcosine by the other. Whereas the methionine carrier had a preference for the L-enantiomorphs, the sarcosine carrier had no stereochemical specificity (Daniels et al.

1969 b). Later work by Nelson and Lerner (1970) showed that there was in avian small intestine a distinct glycine transport system different from the methionine transport site which, like the latter, showed a preference for the L-amino acids.

In tumour cells, however, a somewhat different situation was found to exist; the D-isomers were transported against their concentration gradient (Christensen et al., 1952b)although there was a preferential accumulation of L-enantiomorphs (Paine and Heinz, 1960). In some instances, the uptake of the D-form exceeded that of the L-form. D-tyrosine in S37 ascites (Chirigos et al., 1962) and D-proline in Ehrlich ascites cells (Christensen et al., 1952 b) were accumulated to a greater extent than their respective L-forms. Similarly in brain slices, the tissue concentration of the D-isomers was found to be greater than that of the medium concentration (Takagaki et al., 1959; Margolis and Lajtha, 1968). Furthermore, the D-isomers may reach a higher distribution ratio than the respective L-isomers as appeared to be the case with alanine and possibly methionine (Blasberg and Lajtha, 1965; Neame and Smith, 1965).

b) Requirement for an amino group

A primary amino group or certain limited types of secondary amino groups seemed to be required for amino acid transport into tissue cells. Sarcosine, a secondary amino acid, was strongly accumulated in tumour cells (Christensen and Riggs, 1956) and was reported to exert a strong inhibitory effect on glycine uptake by such cells (Heinz and Walsh, 1958; Paine and Heinz, 1960). On the other hand, N, N-dimethyl

glycine was found to be slightly accumulated by Ehrlich cells (Christensen and Riggs, 1956) while its effects on glycine uptake were negligible (Paine and Heinz, 1960). The secondary amino acids, N-ethyl glycine, N-propyl glycine (Paine and Heinz, 1960) or N-acetylglycine (Heinz and Walsh, 1958) have no effect on glycine uptake, responses which could be explained on the basis of steric effects. The uptake of glycine by hamster small intestine has been found to be inhibited by alanine; sarcosine, N, N-dimethylglycine or betaine had little or no effect (Lin et al., 1962). The requirement for a free amino group in intestinal transport has also been reported by Hajjar and Curran (1970). They studied the effect of the replacement of the amino group by other groups some of them were charged and indicated that such replacement markedly reduced the affinity for the transport site. The latter investigators were also able to show that the binding energy from the amino group was twice that obtainable from the carboxyl group or the side chain.

It is apparent that an optimum distance exists between the amino group and carboxyl group for maximum affinity for the transport site. The inhibition of α-amino acid accumulation in brain slices has been found to be most effective when the amino group was on the α - carbon atom of the amino acid inhibitor (Blasberg and Lajtha, 1965). These workers have demonstrated that β-alanine and GABA showed a diminishing inhibition as the amino group was positioned further from the α-carbon atom. It has also been observed in rat intestine that increased separation between the carboxyl and amino groups in-

creased the inhibition of the sarcosine system but reduced the inhibition of the methionine system (Daniels et al., 1969 a).

c) Modifications of the carboxyl group

Removal of the charge on the carboxyl group of histidine by the formation of an ester or the reduction of the carboxyl group to an alcohol resulted in complete loss of its ability to be actively transported by hamster intestinal preparations (Lin et al., 1962). In contrast is the observations of Evered and Randall (1962), namely that glycine methyl ester was transported against its concentration gradient in rat intestine. This has led Spencer et al., (1966) to extensively investigate the role of the carboxyl group in amino acid transport using the hamster everted small intestine. They used various charged and uncharged replacements of the carboxyl group and have indicated that the carboxyl group must be present for transport against a concentration gradient. Later work by Hajjar and Curran (1970) confirmed the importance of the carboxyl group in amino acid transport across the mucosal border of the rabbit ileum but the requirement for this group was not apparently absolute since compounds lacking the carboxyl group have an affinity for the transport site and are expected to be transported rather slowly when present in high concentration. The affinity for glycine transport system in Ehrlich ascites cells has been found to be dependent on the presence of intact carboxyl group (Paine and Heinz, 1960). Esterification, reduction, or replacement of carboxyl group by sulfonate group, produced no depression of glycine influx.

d) Importance of the α-hydrogen

The ability of the Ehrlich ascites tumour cells to accumulate amino acids has been reported to be increased when α-hydrogen of the transported amino acid was substituted by a methyl group; L, α-methyl tyrosine was concentrated by cells more effectively than was L-tyrosine (Chirigos et al., 1962). An incongruent finding was reported for by Lin et al. (1962) who showed that the possession of an α-hydrogen was not a necessary condition for active transport of amino acids by the intestine but replacement of this hydrogen by methyl group significantly reduced the rate of transport of the compound. Later work has demonstrated a requirement for α-hydrogen; α-aminoisobutyric acid has been found to have no effect on glycine uptake (Matthews and Laster, 1965; Nelson and Lerner, 1970). However on histidine uptake by the rat intestine, α-aminoisobutyric acid exerted a depressant effect and if this could be taken to indicate combination with the site, then the α-hydrogen is not essential for the transport (Finch and Hird, 1960).

e) Effects of the side chain

A wide variety of amino acids with neutral side chains have been found to be actively transported by the intestine (Wilson, 1962). The introduction of a charge into the side chain abolished the affinity for the carrier whether the charge may be positive or negative (Lin et al., 1962). These studies and those of Matthews and Laster (1965) have shown that in hamster intestine the affinity for transmural transport increased as the number of carbon atoms in the side chain

increased. A somewhat similar observation was reported to be the case for amino acid uptake by segments of rat intestine (Finch and Hird, 1960) and for rabbit ileum (Hajjar and Curran, 1970; Peterson et al., 1970). This has led to the suggestion that the lipophilic character of the side chain plays a significant role in the transport process. However, the above relation was determined mainly for amino acids with methyl or methylene groups in the side chain. Tyrosine, histidine and mimosine had a relatively low affinity while methionine had an unexpectedly higher affinity (Hajjar and Curran, 1970). The transport of methionine in rat intestine was effectively inhibited by amino acids with increased length of the side chain (Daniels et al., 1969 a).

III. METHODS AND MATERIALS

III. METHODS AND MATERIALS

1. Tissue Preparation

Adult male New Zealand rabbits weighing 2 to 3 Kg were killed by a blow on the neck and the urinary bladder quickly excised. The detrusor portion of the urinary bladder was then dissected free of surrounding tissues, opened longitudinally and immersed in Krebs solution at 37°C. The mucosa was removed as rapidly as possible by careful dissection through the plane of cleavage between the mucosa and the muscle layer. Fig. 2 shows histological examination of the dissected bladder tissue and revealed proper separation of the mucosa from the underlying muscle layer. The remaining muscle layer was then cut into 10 to 14 pieces and allowed to equilibrate for one hour at 37°C in Krebs solution gassed with 95% oxygen and 5% carbon dioxide.

2. Measurement of Tracer Uptake

After the preincubation period, the tissues were transferred on fine hooks, two to a hook, to test tubes containing Krebs solution 5 ml to which had been added a [^{14}C] labeled compound in minute concentration. The tissues were then incubated further for varying time periods. At the end of the period of incubation, the tissues were removed, blotted on filter paper, rinsed rapidly in Krebs solution and reblotted. This last procedure was aimed at removing surface adherent radioactivity. The tissues were then placed in preweighed scintillation vials and weighed and were then dissolved using NCS solubilizer (a quaternary ammonium base obtained from Amersham/Searle Corp.) in the amount of 1 ml/100 mg tissue weight at 37°C for at least

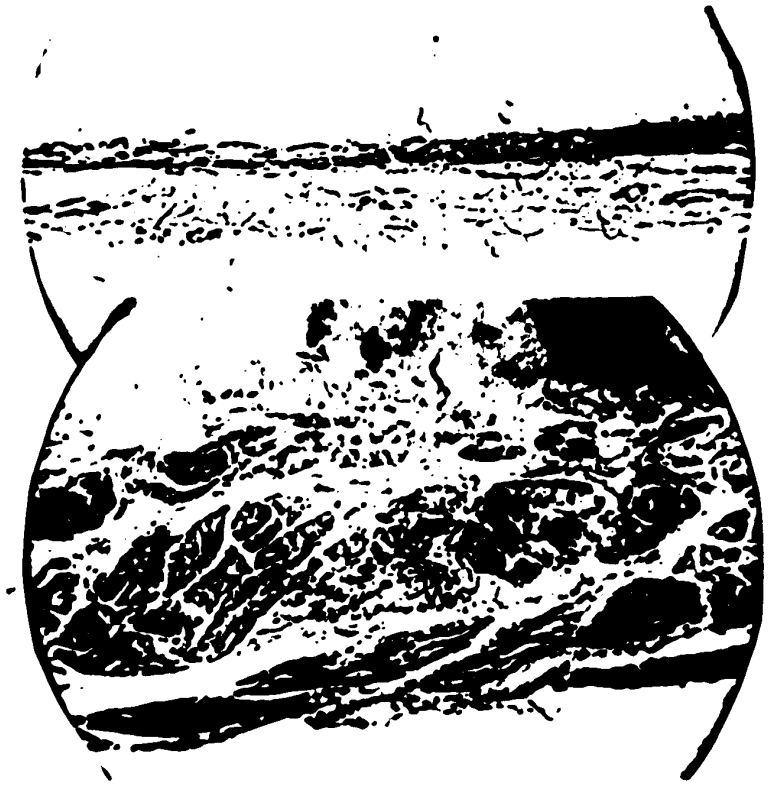


Figure 2. Histological sections of bladder tissue after separation of the mucosa from the muscle layer. Uoper section is the mucosa, lower section is the muscle layer. Sections were stained with Hx. and oesin.

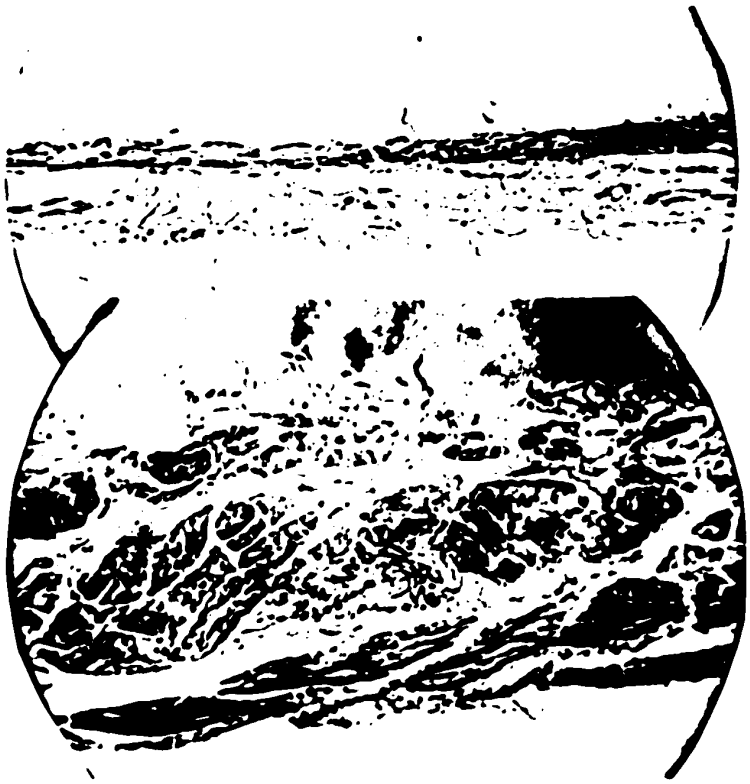


Figure 2. Histological sections of bladder tissue after separation of the mucosa from the muscle layer. Upper section is the mucosa, lower section is the muscle layer. Sections were stained with Hx. and oesin.

6 hours. When tissue dissolution was complete, Bray's phosphor (Bray, 1960), 10 to 15 ml/vial was added and the total [^{14}C] content measured after the vials had been cooled and dark adapted. Duplicate 1.0 ml portions of media were counted for total [^{14}C] after the addition of Bray's phosphor 10 to 15 ml. The total [^{14}C] content was counted using a Picker Nuclear Liquid Scintillation Spectrometer (Liquimat 110) with a counting efficiency for [^{14}C] of about 95%. All vials were counted for a period sufficient to give a precision of statistical error of counting of 1% or less.

2. Measurement of Tracer Efflux

Tissues were incubated in Krebs solution containing a tracer concentration of the radioactive material for 2 hours, then removed, blotted, rinsed rapidly and reblotted and transferred on fine hooks to a series of test tubes containing tracer-free Krebs solution 10 ml at 37°C. Efflux of tracer was then followed for 6 hours. At the end of this period, the total [^{14}C] content remaining in the tissues as well as that present in all efflux material was measured as described above. By addition the total amount of [^{14}C] present in the tissue at time zero was determined. It was assumed that the [^{14}C] tracer was released from a number of compartments exponentially. The disintegration/min in the tissue at time t [f(t)] will thus be given by the equation

$$f(t) = \sum_{i=1}^{n} A_i e^{-K_i t} + B$$

where A_i represents the compartment size

K_i is the efflux rate constant

n is the number of compartments, and

B represents bound fraction from which there is no appreciable efflux during the duration of the experiment.

It should be mentioned that in the above equation all exponentials were assumed to be separated and unrelated. As this does not occur in biological systems, appropriate corrections should be done (Huxley, 1960). The size of the bound fraction was determined using the method of Dick and Lea (1964). The principle of the method is that if the last protion of the curve (at large values of t) was the sum of an exponential plus a bound fraction, then

$$f(t) = Ae^{-Kt} + B$$

Differentiating with respect to "t" we get

$$\frac{df(t)}{dt} = -KAe^{-Kt}$$

$$= -K\,[f(t) - B]$$

$$= KB - K\,f(t)$$

By drawing $\frac{df(t)}{dt}$ vs. f(t) on normal graph paper a straight line is obtained whose x-intercept represents the amount of the bound fraction (B). This is because when $\frac{df(t)}{dt} = 0$, then $KB - K\,f(t) = 0$ or

$f(t) = B$. If the last portion of the curve does not contain a bound fraction, then, when $\frac{d\ f(t)}{dt} = 0$, then $f(t)$ will be equal to zero and the line will pass through the origin. A bound fraction if present should be subtracted from the whole data and the curve redrawn. The last portion of the curve where only one component remains will appear as a series of points to which a straight line may be fitted. The straight line segment is extended back to the zero time and the subtraction or peeling off procedure is begun. The volume of compartment is read from the graph as the intercept of the extended line at time zero. The half-life (t 1/2) is also read from the graph and assuming the efflux is a first order process, then the rate constant (K) can also be calculated from the equation

$$K = \frac{0.693}{t\ 1/2}$$

The peeling off process is repeated until the entire curve has been analyzed. In addition to the graphical method, a computer programme written for the APL/360 system (Cook and Taylor, 1971) has been used for analysis of these efflux data.

4. Correction of Counting Efficiency

Of all the methods used for determination of counting efficiency (Wang and Willis, 1965), the channels ratio technique adopted by Baille (1960) was selected for the purpose of our study. An advantage of this method is that the correction curves produced from chemically quenched standards can also be applied to coloured samples (Baille,

1960; Bush, 1963; Moujaim et al., 1971). However in the case of strongly coloured materials (as in experiments in which 1 ml of 2,4-dinitrophenol containing radioactive solution was counted) the quench correction curve generated from chemically quenched standards is no longer applicable. In such cases the percentage error was calculated by counting samples containing the same abount of radioactivity before and after addition of 2,4-dinitrophenol in amount that produced the same degree of colouration as in our experimental samples. This percentage deviation was used in calculating the final dpm of 2,4-dinitrophenol containing radioactive solutions.

The channels ratio technique is based on adjusting the discriminator settings of the two counting channels so that the statistical error of counting is minimized. In addition a plot of efficiency versus channels ratio yields a useful slope over the desired range of efficiencies and is nearly linear. One channel covered the whole spectrum while the second one covered nearly 1/3 of the spectrum. A calibration curve was constructed by counting a series of samples of varying degrees of quenching but containing a known amount of radioactivity, and the observed counting efficiency was plotted against the ratio of the counting rates in the two channels.(Fig. 3). The experimental samples were then counted under identical instrumental conditions. After subtraction of background activity, the channels ratios were calculated and the corresponding efficiencies read from the graph.

5. Background Activity

The corrections for the counts arising from the unlabeled de-

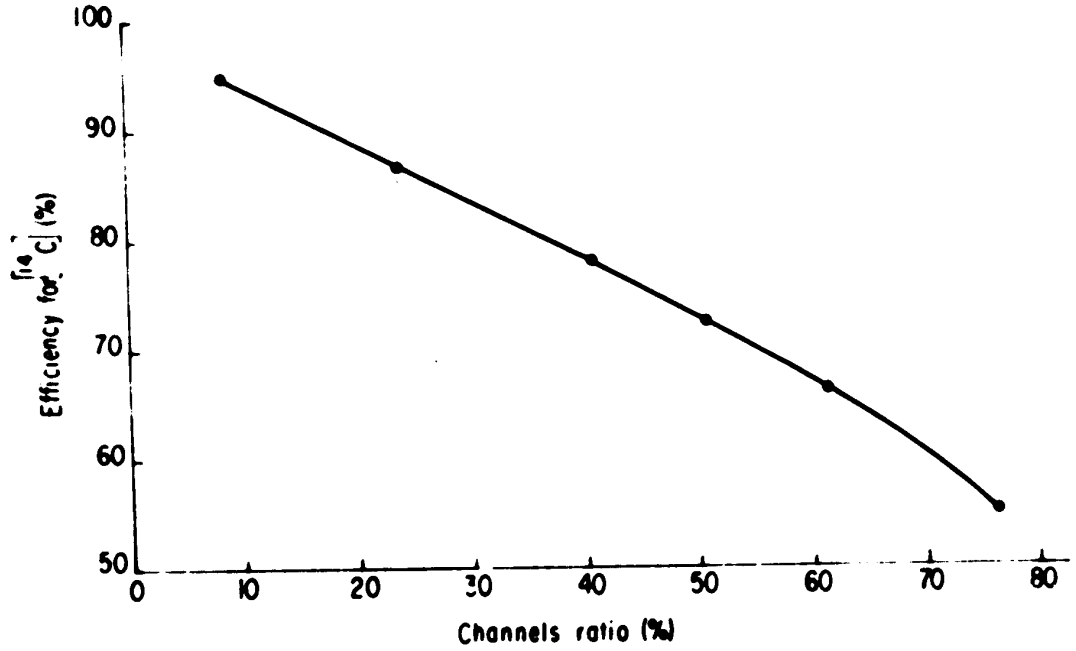


Figure 3. Quench correction curve for [^{14}C] using the channels ratio technique. A series of [^{14}C] quenched standards were counted in two channels; channel A covered the range from 50-850 and channel B from 50-380. These numbers are arbitrary and only represent the energy of the β-particle. Channels ratio was calculated by dividing the cpm in Channel B by the cpm in Channel A.

trusor muscle were determined from a constructed-graph correlating the weight of the bladder tissue to the amount of its background radiation. In cases of the incubation medium, the background activity was determined for each set of experiments.

6. Determination of H_2O, Na^+ and K^+ contents of tissues

After weighing, the tissues were dried in an oven at 105°C for 48 hours and weighed to obtain the total tissue water determined as the difference between the wet and dry weight. 0.1 ml concentrated nitric acid and 0.05 ml 35% hydrogen peroxide solution were added to each of the samples and the samples were then transferred to a sand bath at 200°C to dry. This treatment was repeated until a whitish residue was obtained. This residue was then dissolved in 25 ml double distilled water and the ion content determined by flame photometry using an EEL flame photometer. Standard curves for Na^+ and K^+ were constructed for each experiment using known but different concentrations of these cations. From these standards curves, the ionic content of the sample could be determined by interpolation.

7. Expression of Results

The spaces occupied by the extracellular markers were expressed in ml/100gm wet weight and were calculated from the ratio:

$$\frac{\text{dpm/100gm wet weight of detrusor muscle}}{\text{dpm/ml incubation medium}}$$

The uptake of α-aminoisobutyric acid was expressed in one of the

following ways:

(a) μmoles/gm wet weight tissue =

$$\frac{\text{dpm/gm weight tissue}}{\text{dpm/}\mu\text{mole } \alpha\text{-aminoisobutyric acid}}$$

(b) distribution ratio (%) =

$$\frac{\text{dpm/ml tissue water}}{\text{dpm/ml incubation medium}} \times 100$$

(c) μmoles/ml intracellular water =

$$\frac{Xt - XeVe}{Vt - Ve}$$

where

Xt = total amount of α-aminoisobutyric acid in tissue (μmoles/gm).

Xe = concentration of α-aminoisobutyric acid in extracellular water (μmoles/ml).

Ve = volume of extracellular water (ml/gm), and

Vt = total tissue water (ml/gm).

8. Solutions

The Krebs solution used had the following composition (mM): NaCl 116; KCL 4.6; $CaCl_2$ 1.5; $MgSO_4$ 1.2; $NaHCO_3$ 22; NaH_2PO_4 1.2; D-glucose 50. The medium was equilibrated with 95% O_2 and 5% CO_2

except when the effects of anoxia were studied; in such cases 95% N_2 was substituted for 95% O_2. All incubations were at 37°C unless otherwise specified. In those experiments where Na^+, K^+, Ca^{2+} or Mg^{2+} were omitted from the solution, isoosmolarity (as measured osmometrically) was maintained by sucrose except when otherwise stated. In Na-free solutions, the solution was buffered with Tris [Tris (hydroxymethyl)aminomethane] and the solution aerated with 100% O_2. All solutions had a pH of 7.2 to 7.4.

9. Chemicals Used

The following compounds were used and obtained from the sources indicated:

a) 1 - [^{14}C] - D-mannitol with a specific activity of 16.8 mC/m mole (Amersham/Searle Corp.).

b) U - [^{14}C] - Sucrose with a specific activity of 10.4 mC/m mole (Amersham/Searle Corp.).

c) Carboxyl - [^{14}C] - inulin with a specific activity of 1.45 to 3.65 mC/gm, molecular weight, 5,000 to 5,500 (New England Nuclear Corp.).

d) Carboxyl - [^{14}C] - dextran with a specific activity of 4.63 mC/gm, molecular weight, 15,000 to 17,000 (New England Nuclear Corp.) (referred to as light-dextran); carboxyl - [^{14}C] - dextran with a specific activity of 0.87 mC/gm, molecular weight, 60,000 to 90,000 (New England Nuclear Corp.) (referred to as heavy-dextran).

e) α-amino [3 - ^{14}C] isobutyric acid with a specific activity

of 2.72 mC/m mole (New England Nuclear Corp.). In all experiments, 10 μM of the labeled amino acid were used. Where higher concentrations of the amino acid were required, the [^{14}C] - labeled compound was diluted with unlabeled α-aminoisobutyric acid obtained from the British Drug House Ltd., Poole, England.

f) Unlabeled amino acids (Mann Research Laboratories, Inc. and Sigma Chemical Company).

g) Bovine testicular hyaluronidase (Mann Research Laboratories, Inc.).

h) 2,4-dinitrophenol (Fischer Scientific Co.)

i) Iodoacetic acid (Eastman Organic Chemicals Co.)

j) Ouabain (Nutritional Biochemicals Corp.)

The labeled compounds were obtained in a radiochemically pure form and radiochemical purity was checked by thin layer chromatography using the proper solvent systems followed by determination of the radioactive spot by autoradiography.

10. Statistical Analysis

The variability of samples is expressed as mean $\pm$ standard error of the mean. The significance of differences between samples was determined using Student's "t" test. The difference was regarded as significant when $p < 0.05$. Kinetic analysis was carried out by plotting 1/v against 1/S, the straight line being fitted by linear regression using an Olivetti desk-top computer.

IV. RESULTS

IV. RESULTS

A. EXTRACELLULAR SPACE

Influence of Tissue Weight

Due to variability of sizes of bladder tissue used in this study, the influence of the size of the pieces of the detrusor muscle on the volume of distribution of [^{14}C] - inulin was determined. Figure 4 shows that changing the weight of the tissue within the range 20 to 100 mg did not result in any significant change in the space occupied by [^{14}C] - inulin when the tissues were incubated for 2 hours in [^{14}C] - inulin containing medium. This result is in agreement with that described previously in rat uterine muscle where Daniel (1963) has reported that changing the weight of the uterine tissue from 40 to 140 mg did not alter the volume of distribution of inulin. However Smith and Segal (1968) in determining the extracellular space in rat kidney cortex slices found that the volume of the extracellular space did not undergo any significant change when using half and quarter slices. Using 1/8 slices, the latter authors observed a significant increase in the extracellular space of up to 37% of wet weight. In all our subsequent experiments, the pieces of tissues used had weights of 30 to 90 mg.

Uptake of [^{14}C] - labeled Compounds

The time course of uptake at 37°C of [^{14}C] - labeled inulin by rabbit detrusor muscle was determined for times up to 8 hours in Krebs solution and is shown in Figure 5. As is apparent from the Figure, the space available within the muscle for the distribution of [^{14}C] -

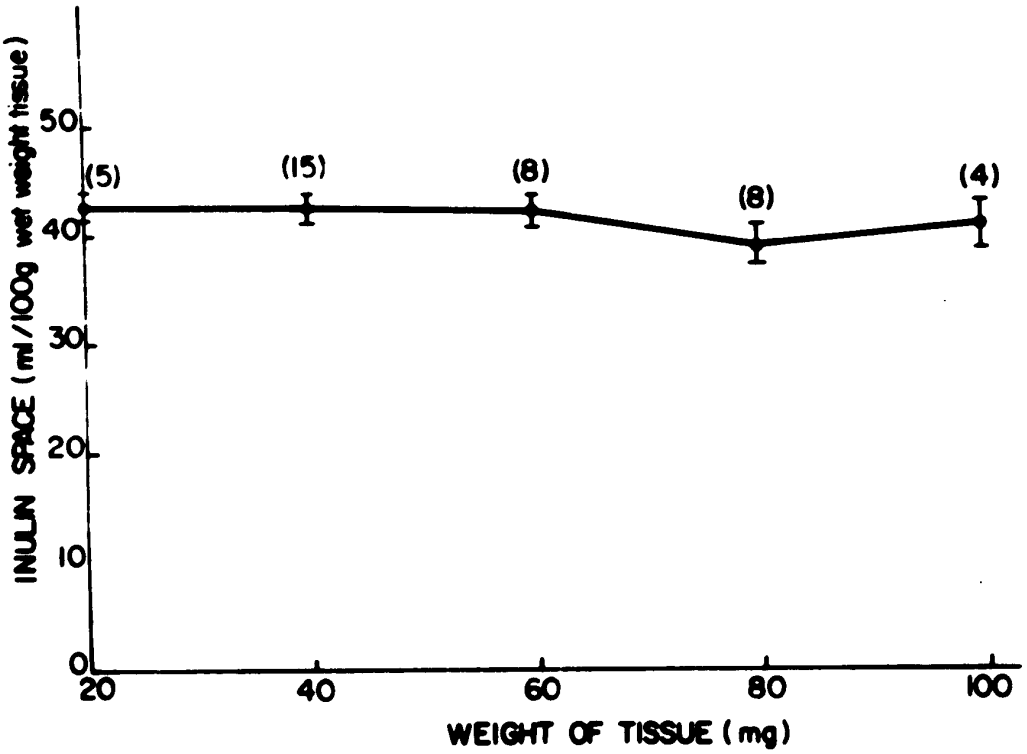


Figure 4. Effect of tissue size on the inulin space of rabbit detrusor muscle. Muscle strips were incubated at 37°C for 2 hr. in Krebs solution containing tracer quantities of [^{14}C]-inulin. Each point is the mean ± S.E. The number of observations is indicated in brackets.

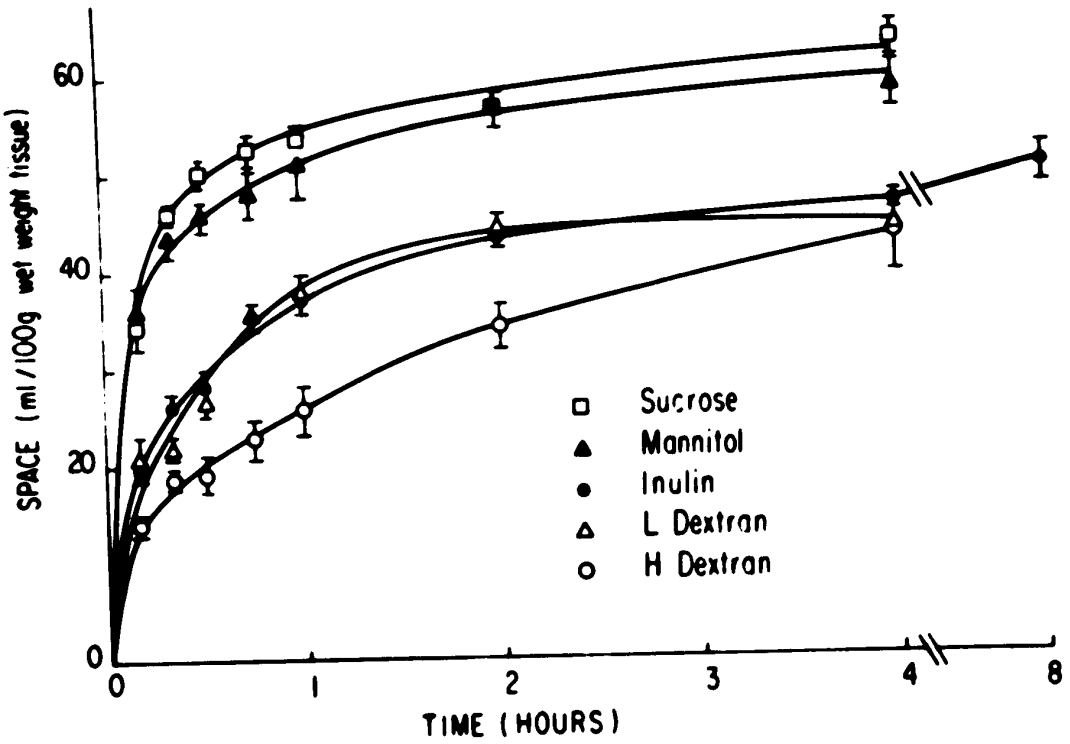


Figure 5. Uptake of extracellular markers by rabbit detrusor muscle. Muscle strips were incubated at 37°C for up to 4 hours in Krebs solution containing tracer quantities of one of the following: [^{14}C]-mannitol Δ—Δ; [^{14}C]-sucrose, □—□; [^{14}C]-inulin, ●—●; [^{14}C]-light dextran, Δ—Δ; and [^{14}C]-heavy dextran, ○—○. Each point is the mean ± S.E. of 6-10 observations.

inulin increased as a function of time. [^{14}C] - inulin equilibrated with about 38% of the wet weight of the tissue by one hour, then over the period between 1 and 4 hours, the [^{14}C] - inulin space increased at a slow rate. The differences between the 1 and 2 hour levels and the 1 and 4 hour levels of the [^{14}C] - inulin space are significant at the 0.05 level while the difference between the 2 and 4 hour levels is insignificant at the 0.05 level. When the incubation period was extended to 8 hours, the volume of distribution of [^{14}C] - inulin increased further and reached a value of 52% of the wet weight of the tissue by that time.

Since inulin is a relatively large molecule with a molecular radius of 15 Å (the hydrated radius being about 30 Å), it may not equilibrate with the total extracellular space due to its molecular size. We therefore investigated the uptake of four other extracellular markers, two of them smaller (mannitol and sucrose) and two (L. dextran and H. dextran) larger than inulin. Figure 5 shows that the influx of [^{14}C] - mannitol was characterized by an initial rapid uptake during the first 30 minutes followed by a plateau which represented about 50% of the wet weight. Upon prolonged incubation, there was a slow increase in the distribution reaching a value of about 60% after 4 hours. The difference between the 1 and 4 hour values is significant at the 0.05 level. The distribution of [^{14}C] - sucrose was similar to that of [^{14}C] - mannitol while that of [^{14}C] - light-dextran was very similar to [^{14}C] - inulin. However the uptake of [^{14}C] - heavy-dextran followed a completely different pattern. Its uptake was slower than any

of the other markers, though after 4 hours its distribution was approximating that of [^{14}C] - inulin and was not significantly different. This might denote that although the space available within the muscle for the distribution of [^{14}C] - inulin and [^{14}C] - heavy-dextran is the same, the latter has a slower rate of diffusion than does the former.

Effects of Hyaluronidase

The observation that the spaces available for [^{14}C] - mannitol and [^{14}C] - sucrose (low molecular weight compounds) were higher than those obtainable for [^{14}C] - inulin, [^{14}C] - light-dextran and [^{14}C] - heavy-dextran (of higher molecular weight) at long equilibration periods suggested that the former substances might gain access to certain regions which are inaccessible to the larger compounds. Hyaluronic acid, a mucopolysaccharide of high viscosity was found to exist between the cells of smooth muscle in guinea-pig taenia coli (Goodford and Leach, 1966) and in such tissues it acts as a cementing substance between the muscle fibres. Hyaluronic acid is broken down by the enzyme hyaluronidase and this reduces the viscosity and allows a greater spreading of materials within the tissue spaces.

For this reason the effect of treating tissues with hyaluronidase on the distribution of these extracellular markers was determined. Table III shows the results obtained at the end of 4 hour incubation period when the tissues had been pretreated for one hour with 50 USP units/ml bovine testicular hyaluronidase. With all the markers, their volumes of distribution was markedly reduced by such treatment.

TABLE III

EFFECT OF HYALURONIDASE ON THE DISTRIBUTION OF SOME EXTRACELLULAR MARKERS BY RABBIT DETRUSOR MUSCLE.

Tissues were preincubated for 60 min at 37°C in Krebs solution with or without added hyaluronidase (50 USP units/ml). Tissues were then incubated for 4 hr in Krebs solution containing one of the extracellular markers. Mean values ± S.E. of 6 observations. All values obtained from hyaluronidase treated tissues are significantly different from non-treated ones.

Extracellular Marker	Control	Hyaluronidase Treatment	Percentage Reduction
Inulin	48.05 ± 1.75	39.97 ± 2.48	17
Mannitol	59.38 ± 2.37	50.39 ± 1.62	15
Sucrose	58.04 ± 1.70	48.78 ± 1.41	16
L-Dextran	46.23 ± 2.68	34.55 ± 0.89	25
H-Dextran	36.75 ± 3.02	28.99 ± 1.17	21

The percentage reduction varied from 15 to 25% with an average of 19%. This result was rather surprising and unexpected and this led us to think that there might be something wrong with the enzyme preparation. Consequently, new batches of the enzyme were used and the results were found to be reproducible. Also, the intensity of Alcian blue-stained sections of detrusor muscle that had been treated with hyaluronidase, was observed to be less than that of non-treated sections, thus indicating the activity of the enzyme on mucopolysaccharides.

Further experiments were designed to demonstrate the effects of increasing the concentration of the enzyme as well as increasing the period of exposure of the tissues to its action. The reason for this is that higher concentrations of the enzyme or long periods of exposure may be required for the enzyme to exert its action. Table IV shows the space occupied by [^{14}C]-inulin at the end of 4 hours in tissues exposed to different treatments with hyaluronidase. In all cases the enzyme was present during the preincubation period. Increasing the concentration of hyaluronidase from 50 to 100 USP units/ml did not alter the trend irrespective of whether the period of preincubation was 60 or 120 minutes. Furthermore the presence or absence of the enzyme during the incubation period did not significantly affect the volume of distribution of [^{14}C]-inulin.

To investigate this problem further we measured the changes in tissue weight, Na^+ and K^+ content of tissues in normal Krebs medium and in media to which have been added 50 or 100 USP units/ml of the enzyme preparation. The enzyme was present during both the preincubation and

TABLE IV

EFFECT OF VARIOUS HYALURONIDASE TREATMENTS ON THE VOLUME OF DISTRIBUTION OF [^{14}C]-INULIN BY RABBIT DETRUSOR MUSCLE.

Tissues were subjected to the treatments shown. [^{14}C]-inulin was added to the incubation media and incubations were continued for 4 hr. Mean values ± S.E. of 5 observations. All values obtained from hyaluronidase treated tissues are significantly different from non-treated ones.

Preincubation Medium	Incubation Medium	[^{14}C]-inulin Space (ml/100 gm wet wt)
Normal Krebs	Normal Krebs	45.63 ± 1.51
Hyaluronidase treatment		
50 units/ml for 60 min.	Normal Krebs	38.18 ± 1.04
50 units/ml for 60 min.	50 units hyaluronidase/ml	35.09 ± 1.19
50 units/ml for 120 min.	Normal Krebs	33.48 ± 1.30
100 units/ml for 60 min.	Normal Krebs	35.65 ± 1.49
100 units/ml for 120 min.	Normal Krebs	37.75 ± 2.18

incubation periods. It was found that in the presence of 50 USP units/ml hyaluronidase the tissue contents of H_2O, Na^+ and K^+ were not significantly different from the control (Table V). However this treatment produced shrinkage of the weight of the tissue which amounted to about 12% compared to the control. The reduction of the space occupied by the extracellular markers may be due to one of the following:

1- Swelling of the cells with no change in tissue volume.

2- Shrinkage of the tissue with no change in cell volume.

3- Combination of the above.

The shrinkage of the tissue observed after hyaluronidase treatment rules out the first possibility. If the difference between the total tissue water and the space occupied by the extracellular marker represents mainly the intracellular water, then the enzyme treatment causes swelling of the cells and consequently the second possibility is not applicable. It therefore seems that a combination of shrinkage of the tissue and swelling of the cells is the most likely explanation. The lack of changes in the Na^+ and K^+ content per unit weight of dry solids after 50 USP units/ml of hyaluronidase could be explained if the shrinkage of the tissue was due to extrusion of water without any accompanying change of tissue solids. However with higher concentration of the enzyme (100 USP units/ml) there was lowering of the tissue Na^+ and K^+ levels when expressed in terms of dry weight. One possibility is that this concentration of the enzyme might affect some of the extracellular binding sites for Na^+ releasing Na^+ in the external medium and thereby lowering its concentration in the tissues. However

TABLE V

EFFECT OF HYALURONIDASE ON TISSUE WEIGHT, WATER AND ELECTROLYTE CONTENT OF RABBIT DETRUSOR MUSCLE.

Tissues were preincubated for 1 hr followed by 4 hr incubation in absence and presence of 50 or 100 USP units/ml hyaluronidase. Data represent the mean ± S.E. of 9 observations. Values marked with an asterisk differ significantly from values obtained in the absence of hyaluronidase.

Tissue Treatment	Final wt. / Initial wt.	Total Tissue Water (ml/100 gm wt)	Na^+ Content (mEq/kg) Wet wt.	Na^+ Content (mEq/kg) Dry wt.	K^+ Content (mEq/kg) Wet wt.	K^+ Content (mEq/kg) Dry wt.
Normal Krebs	0.94 ± 1.8	85.35 ± 0.47	83.54 ± 1.36	572 ± 18.8	44.36 ± 1.86	303 ± 8.8
Normal Krebs + 50 units/ml hyalase	0.83* ± 1.5	86.12 ± 0.54	80.87 ± 0.95	589 ± 22.3	41.01 ± 1.13	297 ± 6.8
Normal Krebs + 100 units/ml hyalase	0.75* ± 0.8	84.00 ± 0.52	80.29 ± 0.83	505* ± 13.3	40.83 ± 1.22	256* ± 7.8

lowering of K^+ was also observed by Goodford and Leach (1966) with higher concentrations of the enzyme but the reason for this is not fully understood.

Efflux of [^{14}C]-labeled Compounds

To test homogeneity of the space occupied by various [^{14}C]-labeled extracellular markers the efflux of these markers from the bladder muscle was performed in preparations which have been preincubated with the tracer for 2 hours. The tissues were then transferred successively to a series of test tubes, each containing 10 ml of normal Krebs solution. This caused efflux of the tracer from the tissues and because of the large volume of the incubation medium, the back influx of the tracer would be negligible. Tissue desaturation curves were then constructed by plotting semilogarithmically the amount of radioactivity remaining in the tissue against time. The tissue desaturation curves obtained for [^{14}C] - mannitol and [^{14}C] - inulin efflux in a single experiment are shown in Figure 6; it can be seen that there was an initially rapid efflux of these compounds followed by a much slower rate of efflux. Subtraction of the bound fraction from the desaturation curves shows clearly however that efflux was proceeding up to 6 hours at which time, the experiment was terminated. It is apparent that the rate of efflux of [^{14}C] - mannitol was considerably faster than that of [^{14}C] - inulin (Figure 6), that efflux of these compounds was exponential and that the efflux was occurring from more than one compartment.

The curve peeling technique used to resolve the tissue desatu-

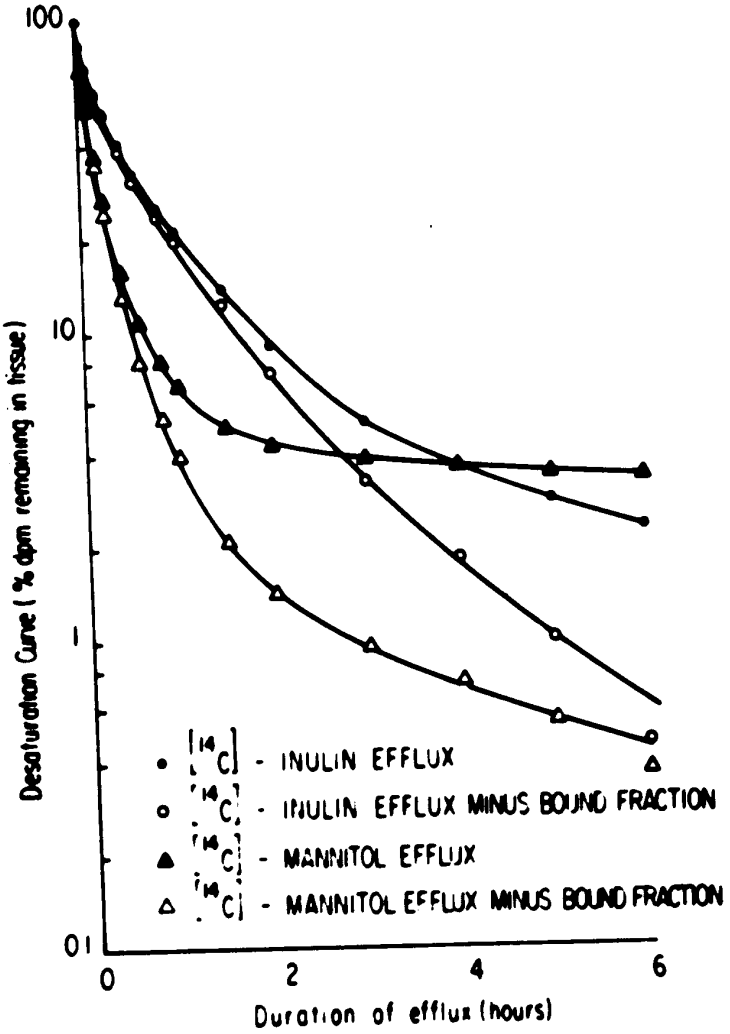


Figure 6. Efflux of [^{14}C]-mannitol and [^{14}C]-inulin from rabbit detrusor muscle. Muscle strips from the same rabbit were incubated at 37°C for 2 hr in Krebs solution containing either [^{14}C]-mannitol or [^{14}C]-inulin. The strips were then placed in tracer-free Krebs solution at 37°C for 6 hr and the efflux of [^{14}C] determined. The semilog plots of tissue radioactivity against time are shown for: [^{14}C]-inulin efflux, ●—●: [^{14}C]-inulin efflux after subtraction of the bound fraction, ○—○; [^{14}C]-mannitol efflux, ▲—▲; [^{14}C]-mannitol efflux after subtraction of the bound fraction, △—△.

ration curve into its exponential components is only valid if the last linear portion of the curve is described by a single exponential term i.e., the tracer is emerging from a single homogeneous compartment. To test whether the efflux was from more than a single compartment, the method of Dick and Lea (1964) was used. By this method, an arithmetic plot of the efflux rate (dpm/m) as the ordinate against the tissue desaturation (dpm) as the abscissa yields a straight line which when extrapolated to zero efflux rate will provide an intercept on the desaturation axis which is equal to the bound fraction (Figure 7). An accurate measure of this bound fraction is essential if one is to obtain a correct values for compartment sizes and rate constants since the latter parameters are very sensitive to small changes in volume of the bound fraction. This was analyzed further by plotting the logarithm of the efflux rate versus logarithm of dpm in the tissue. Keynes and Swan (1959) and Persoff (1960) have pointed out that a slope of one will be obtained if the efflux of the tracer was from a single homogeneous compartment while efflux from more than one compartment yields a slope greater than unity. Figure 8 shows log-log plot of tissue radioactivity against efflux rate for [^{14}C]-inulin and the effect of subtraction of the bound fraction. The slope of the terminal portion of the original curve was 2.4 while after subtraction of the bound fraction, the slope became closer to one. After subtraction of the bound fraction, the resulting semilog plot of the tissue desaturation against time was examined and the number of compartments determined by curve peeling using the graphical method and a computer programme written for the APL/360 system (Cook and Taylor, 1971).

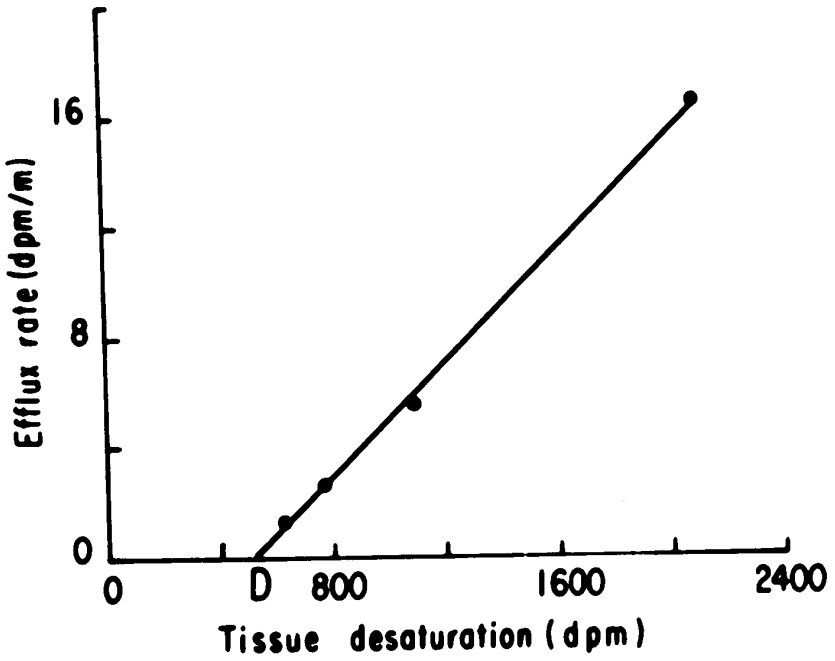


Figure 7. Illustration of the method used to determine the 'bound' fraction "D" from plot of tissue desaturation (dpm) against efflux rate (dpm/m) of the terminal portion of [^{14}C]-inulin efflux curve.

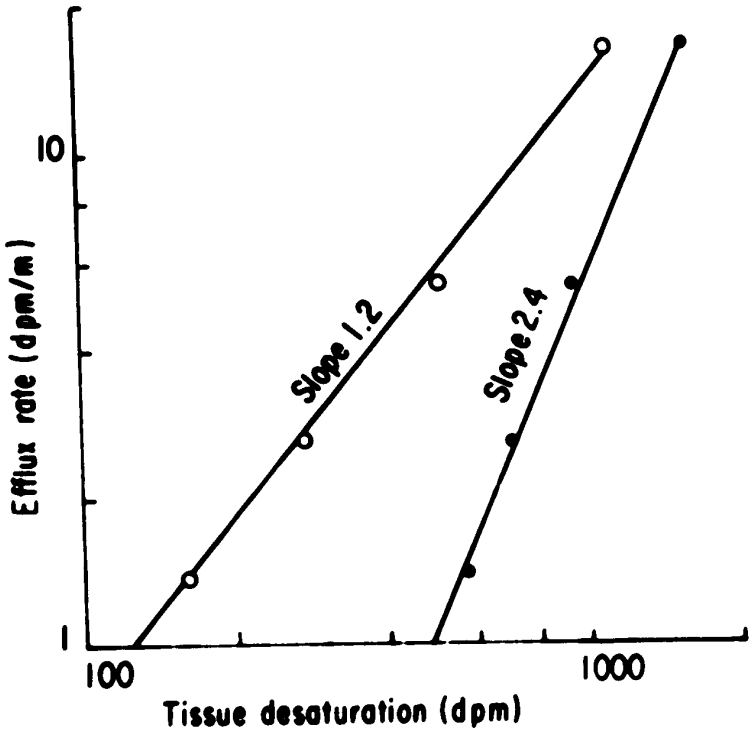


Figure 8. A log-log plot of tissue radioactivity for [^{14}C]-inulin (dpm) against efflux rate (dpm/m) of the final portion of the efflux curve before and after subtraction of the bound fraction.

One study, illustrated in Figure 9, shows the compartmental analysis of the efflux of [^{14}C]- inulin. Curve A is the plot of efflux after subtraction of the bound fraction. The linear portion of the curve at large values of "t" was used to obtain the size of the slowest compartment. The contribution of this compartment was subtracted from the plot and this resulted in a further desaturation curve (curve B, Figure 9) which was linear at the latter time periods of efflux. From this portion of the curve, the size of the second slowest compartment was determined. The contribution of this compartment was then subtracted from the remaining points, resulting in a further desaturation curve (Curve C, Figure 9), which was linear. Using the aforementioned method it was found that the desaturation curves for mannitol, sucrose and inulin could all be resolved into three compartments and a bound fraction.

The above analysis assumes that the three exponential terms are physically independent. As this is not encountered in biological systems, Huxley (1960) has proposed a method to correct for the interactions between these compartments. He assumed that the bathing solution, extracellular and cellular fluids are arranged in series and he showed that extrapolation of the cellular compartment of efflux to zero time leads to an overestimation of this fraction. He corrected for this slow fraction by applying the formula

$$P_{30} = \frac{AB\,(K_1 - K_2)^2}{AK_1^2 + BK_2^2}$$

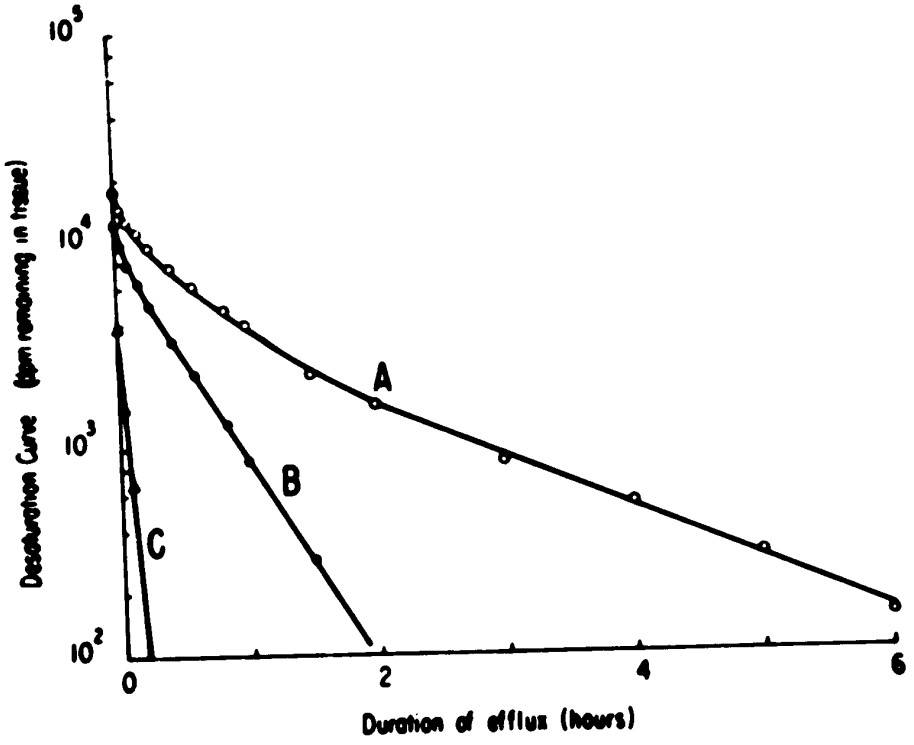


Figure 9. Compartmental analysis of efflux of [^{14}C]-inulin from rabbit detrusor muscle. Efflux of [^{14}C]-inulin was determined as in Fig. 3. The semilog plots of tissue radioactivity against time are shown for: A, O—O, the plot of the total efflux minus the bound fraction; B, ●—●, the plot of the efflux remaining after the subtraction of the slowest compartment from A; and, C, Δ—Δ, the plot of the efflux remaining after the subtraction of the intermediate compartment from B.

where P_{30} is the corrected slow fraction

A represents the volume of the fast component

B represents the volume of the slow component

K_1 is the rate constant for the fast fraction, and

K_2 is the rate constant for the slow fraction.

In our system, it was assumed that the slowest compartments (B,C), the fast one (A) and the external medium are arranged in series (Figure 10) and the Huxley correction was applied to them. The corrected fast fraction (A cor) was then calculated from the equation

$$A\ cor = Y_o - B\ cor - C\ cor - D$$

where Y_o is the sum of volumes of the compartments at time zero.

The results of this method of analysis with the compartment sizes, rate constants and half times for inulin, mannitol and sucrose are presented in Table VI. From this table it can be seen that the fast compartment of [^{14}C] - mannitol and [^{14}C] - sucrose is equal to the total space occupied by [^{14}C] - inulin. If the difference between the volume of distribution of [^{14}C] - inulin and that of [^{14}C] - mannitol or [^{14}C] - sucrose is due to intracellular penetration by the latter substances, then [^{14}C] - inulin space is an approximate measure of the true extracellular space.

Tissue Contents of Na^+ and K^+

The tissues contents of H_2O, Na^+ and K^+ of detrusor muscle strips were determined after 240 minutes incubation in Krebs solution

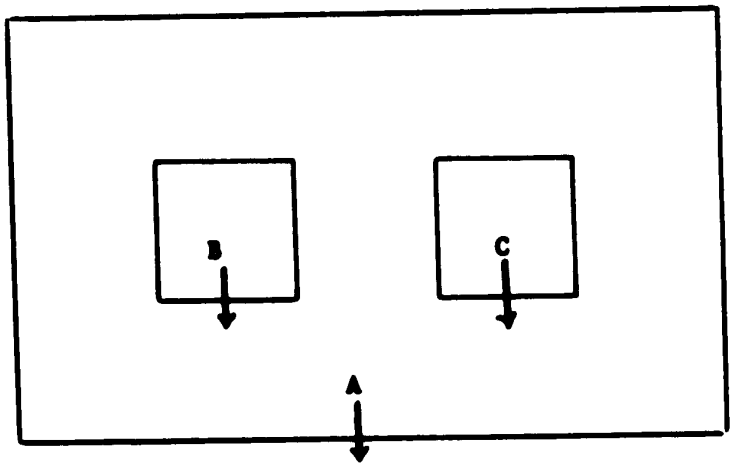


Figure 10. Schematic representation of the various compartments to which Huxley corrections were applied.

TABLE VI

COMPUTER ANALYSIS OF EFFLUX OF SOME EXTRACELLULAR MARKERS FROM RABBIT DETRUSOR MUSCLE.

Compartment		Inulin	Mannitol	Sucrose
Fast	Acor	19.2 ± 1.8	43.6 ± 7.2	38.4 ± 3.9
	t1/2	3.8 ± 1.2	5.7 ± 2.7	4.5 ± 1.2
	K	0.2379 ± 0.0636	0.2208 ± 0.0838	0.1883 ± 0.0464
Medium	Bcor	10.4 ± 3.6	12.9 ± 5.6	11.7 ± 3.9
	t1/2	28.4 ± 8.5	20.7 ± 7.7	17.4 ± 2.5
	K	0.0315 ± 0.0076	0.0445 ± 0.0101	0.0419 ± 0.0051
Slow	Ccor	11.5 ± 1.7	2.5 ± 0.42	3.2 ± 0.48
	t1/2	60.0 ± 8.1	98.6 ± 13.9	96.7 ± 12.6
	K	0.0127 ± 0.0014	0.0074 ± 0.0009	0.0075 ± 0.0008
Bound D		0.8 ± 0.08	1.4 ± 0.14	3.3 ± 0.29

at 37°C and are shown in Table VII. The intracellular concentrations of Na^+ and K^+ were then calculated using as estimations of the volume of the extracellular space, the spaces occupied by the [^{14}C]-labeled compounds at equilibration (see Table VII). It can be seen that the calculated intracellular concentrations of Na^+ and K^+ varied between 6 to 55 mEq/L and 102 to 184 mEq/L respectively depending on the volume used for the extracellular space (Table VII). It is important to note that when sucrose was used as the extracellular marker, the calculated intracellular Na^+ content was negative. It is evident that the spaces occupied by inulin and dextran and by mannitol and sucrose were about 50% and 70% of the total tissue water respectively.

Factors Affecting the Uptake of [^{14}C]-labeled Compounds

Some of the factors known to affect the transport processes across the cell membrane have been studied for their effects on the uptake of extracellular markers in an attempt to determine which of the markers is suitable for our purposes. A non-penetrating marker is one whose uptake is not changed when the cellular barriers are damaged.

1. Effect of Temperature

The time course of uptake of [^{14}C] - inulin and [^{14}C] - mannitol by the bladder muscle at 2°C and 37°C is shown in Figure 11. It can be seen that the uptake of [^{14}C] - inulin at 2°C is less than that at 37°C but as the incubation period was prolonged the percentage space obtained at 2°C is approaching that at 37°C. This suggests that the difference in uptake these two temperatures is a difference in the

TABLE VII

DETERMINATION OF INTRACELLULAR Na^+ and K^+ CONTENT OF RABBIT DETRUSOR MUSCLE.

The calculations of intracellular Na^+ and K^+ contents were based on determinations of extracellular space and tissue H_2O, Na^+ and K^+ contents made after incubation in Krebs solution at 37°C for 240 min. Total tissue H_2O = 85.35 ml/100 gm wet weight; total tissue Na^+ = 83.54m moles/kg wet weight; total tissue K^+ = 44.36m moles/kg wet weight.

Extracellular Marker	% Space (ml/100gm)	Intracellular Electrolyte Content (mM)	
		Na^+	K^+
Inulin	45.12 ± 0.74 (43)	51	105
Mannitol	58.53 ± 1.06 (31)	6	155
Sucrose	62.90 ± 3.07 (6)	-20	184
L-Dextran	43.68 ± 1.36 (27)	53	102
H-Dextran	43.04 ± 3.84 (6)	55	100

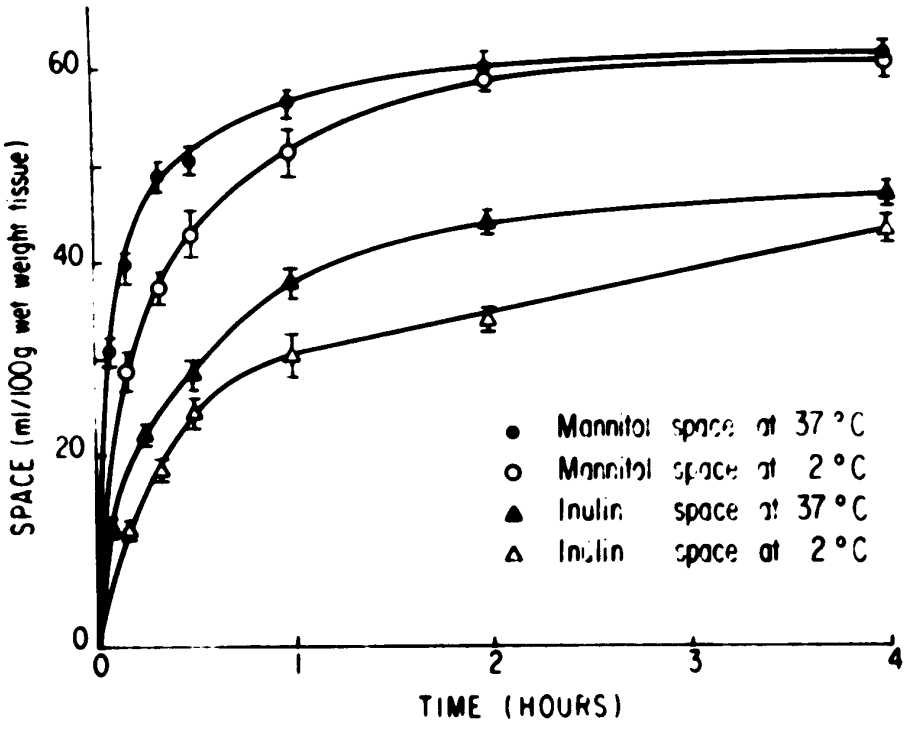


Figure 11. **Effect of temperature on uptake of inulin and mannitol by rabbit detrusor muscle. Tissues were incubated in Krebs solution containing tracer quantities of [^{14}C]-inulin or [^{14}C]-mannitol at either 37°C or 2°C. Mean values ± S.E. of 6 observations.**

rate of diffusion which is slow at low temperature compared to that at high temperature. A similar pattern of uptake was also observed for [^{14}C] - mannitol; the volumes of distribution at these two temperatures reached similar values at the end of a 2 hour incubation period. This shows that the spaces available within the muscle for [^{14}C] - inulin and [^{14}C] - mannitol are the same at 2°C and at 37°C.

2. Effect of Metabolic Inhibitors

In order to simulate a condition of metabolic stress, the tissues were preincubated for 40 minutes in glucose-free medium containing DNP and IAA, 1 mM each, followed by 20 minutes in glucose-free medium plus the tracer for further 4 hours. Table VIII shows that the uptake of low molecular weight compounds, [^{14}C] - mannitol and [^{14}C] - sucrose was greatly increased after such treatment. On the other hand, the uptake of [^{14}C] - inulin and [^{14}C] - dextran was not significantly changed. In these experiments, however, the tissues were incubated in glucose-free media; if [^{14}C] - mannitol and [^{14}C] - sucrose could gain access into the cells by the same transport system as that of glucose, then one might expect that in absence of glucose, the uptake of [^{14}C] - mannitol and [^{14}C] - sucrose to be increased due to a lack of competition. This possibility was investigated in more detail and the uptake of [^{14}C] - mannitol under varying conditions is presented in Table IX. From the table it can be seen that the uptake of [^{14}C] - mannitol as well as the tissue weight were not significantly different whether or not glucose was present. Furthermore, the absence of glucose has no apparent effect on the response to IAA or to DNP and IAA.

TABLE VIII

EFFECT OF METABOLIC STRESS ON THE UPTAKE OF [^{14}C]-LABELLED EXTRACELLULAR MARKERS BY RABBIT DETRUSOR MUSCLE.

Tissues were preincubated at 37°C for 30 min in glucose-free Krebs solution containing 2.4 dinitrophenol (DNP) and iodoacetic acid (IAA), 1 mM each. They were then transferred to glucose-free Krebs solution for 20 min. The tracer was then added and tissues were incubated for 4 hr. Data represent the mean ± S.E. of 6 observations. Values marked with an asterisk differ significantly from control values.

Extracellular Marker	Space (ml/100 gm wet weight tissue	
	Control	Glucose free + IAA + DNP
Mannitol	59.4 ± 2.37	77.4* ± 1.34
Sucrose	58.0 ± 1.70	75.2* ± 1.13
Inulin	48.0 ± 1.75	51.9 ± 2.61
L-Dextran	46.2 ± 2.68	47.2 ± 2.67
H-Dextran	36.8 ± 3.02	37.6 ± 1.05

TABLE IX

EFFECT OF GLUCOSE AND METABOLIC INHIBITORS ON THE UPTAKE OF [^{14}C]-INULIN BY RABBIT DETRUSOR MUSCLE.

Tissues were preincubated at 37°C in Krebs or glucose-free Krebs solution containing 1 mM dinitrophenol (for 30 min) or 1 mM iodoacetic acid (for 30 min) or both 1 mM dinitrophenol and iodoacetic acid (for 40 min). Tissues were incubated for 4 hr at 37°C in fresh media containing tracer amounts of [^{14}C]-mannitol and 1 mM dinitrophenol if this inhibitor had been present alone during preincubation. Mean values ± S.E. of 8 observations. Values marked with an asterisk differ significantly from the control.

Tissue Treatment	$\frac{\text{Final wt.}}{\text{Initial wt.}}$	[^{14}C]-mannitol space (ml/100 gm)
Normal Krebs (N.K.)	0.93 ± 0.018	58.4 ± 1.18
N.K. + DNP	0.95 ± 0.030	55.2 ± 1.29
N.K. + IAA	1.05* ± 0.023	68.5* ± 2.18
N.K. + DNP + IAA	1.28* ± 0.023	77.3* ± 0.95
Glucose-free Krebs (G.F.)	0.94 ± 0.019	59.7 ± 1.02
G.F. + DNP	1.24* ± 0.023	75.5* ± 0.71
G.F. + IAA	1.06* ± 0.011	67.4* ± 1.21
G.F. + DNP + IAA	1.18* ± 0.011	78.9* ± 1.02

However, the greatest effect was noticeable with DNP alone where omission of glucose from the external medium caused marked swelling of the tissue and an increase in the space occupied by [^{14}C] - mannitol.

3. Effect of Cations

Swelling of tissues was observed when they were incubated in media deficient in Ca^{++} whereas omission of K^{+} and Mg^{++} from the incubation medium produced no significant effect on tissue weight (Table X). Although the space occupied by [^{14}C] - inulin was not significantly changed in the absence of K^{+} and Ca^{++}, there was a slight reduction in the absence of Mg^{++}. On the other hand, lack of Na from the incubation media produced the most drastic changes in the tissue weight, total tissue water and [^{14}C] - inulin space. The tissue weight was reduced by about 40%, tissue water by 12% and space available for [^{14}C] - inulin by 30%. Since sucrose was the only substituent used for Na^{+}, a sucrose effect could not be excluded.

TABLE X

EFFECT OF CATIONS ON TISSUE WEIGHT, TOTAL WATER CONTENT AND [^{14}C]-INULIN SPACE IN RABBIT DETRUSOR MUSCLE.

Tissues were preincubated for 60 min at 37°C in Krebs solution or Na^+-free, or K^+-free or Ca^{2+}-free, or Mg^{2+}-free Krebs solution. [^{14}C]-inulin was then added and incubation continued for 2 hr. Mean values ± S.E. of 6 - 8 observations. Values marked with asterisk differ significantly from control values.

Cation Omitted	$\frac{\text{Final Wt.}}{\text{Initial Wt.}} \times 100$	Total Tissue Water(ml/100gm)	[^{14}C]-inulin Space(ml/100gm)
None	98.1 ± 2.8	87.8 ± 0.61	43.7 ± 2.3
Sodium	59.2* ± 1.1	77.7* ± 0.38	30.7* ± 2.1
Potassium	97.8 ± 1.7	85.4 ± 0.52	41.0 ± 2.4
Calcium	110.2* ± 2.5	89.0 ± 0.42	40.1 ± 2.4
Magnesium	95.0 ± 3.0	87.5 ± 0.34	38.7* ± 0.9

CONCLUSION

These studies confirmed the existence of many problems in obtaining an exact value for the extracellular space in detrusor muscle. To obviate these problems, calculation of the uptake of α-aminoisobutyric acid will be expressed either in terms of its distribution between the tissue water and external medium or in terms of unit weight. No attempt will be made however to calculate the intracellular content of the amino acid save if need be as in kinetics and competition studies. In such cases the value for the inulin space at 2 hours will be taken as a measure of the extracellular space (i.e., 43 ml/100 gm wet weight). This decision is based on the following observations:

1. The results of compartmental analysis have shown that the size of the fast fraction of mannitol and sucrose is nearly the same as that of the total volume of distribution of inulin. Assuming that the fast fraction of mannitol and sucrose is the one emerging from the extracellular space, then the inulin space could be considered as an approximation of the extracellular space volume.
2. Burnstock et al. (1963) have reviewed the available data for the intracellular K^+ content of a variety of mammalian visceral smooth muscles and have reported that for equilibrated preparations the most likely value is around 100 mM. If this value holds true for the rabbit detrusor muscle, then a value of 43 ml/100 gm tissue is an approximate measure of the extracellular space.

3. Factors which are known to damage the cellular barriers did not increase the volume of distribution of inulin suggesting that it does not go into the cells and its distribution is entirely extracellular.

It must be emphasised that the assumptions made in such studies were introduced because they best satisfy our current knowledge and in the absence of definite information about the system allow assessment of our results. Everyone of these assumptions is nevertheless open to criticism.

B. AMINO ACID TRANSPORT

Influence of Tissue Size on Uptake of α-aminoisobutyric acid

Smith and Segal (1968) have shown that careful attention should be paid to the influence of size on amino acid uptake by isolated tissues since they found significantly higher accumulation of both dibasic and mono-amino-mono-carboxylic amino acids by small kidney segments; the increase was particularly marked when the incubation period was prolonged. In the present study changing the weight of the pieces of detrusor muscle used from 10 to 110 mg did not alter the uptake of α - aminoisobutyric acid in tissues incubated for 2 hours (Figure 12). In all subsequent studies only pieces of tissue weighing 30 to 90 mg were used.

Time Course of Uptake

The first step in studying the transport of α-aminoisobutyric acid in detrusor muscle was to investigate the time course of uptake to determine whether the uptake was saturable or not, and whether it was an uphill transport process. Figure 13 shows the effect of time on the uptake of 10 μM and 10 mM of α-aminoisobutyric acid expressed as the distribution ratio. The uptake of 10 μM α-aminoisobutyric acid increased in an approximately linear fashion from 10 to 120 minutes with approximately steady state conditions being reached after about 4 hours at a distribution ratio of about 350%. The uptake of amino acid was against the concentration gradient (i.e., uphill transport) after about 30 minutes when a distribution ratio of 100% was achieved.

The distribution ratio of 10 mM α-aminoisobutyric acid was

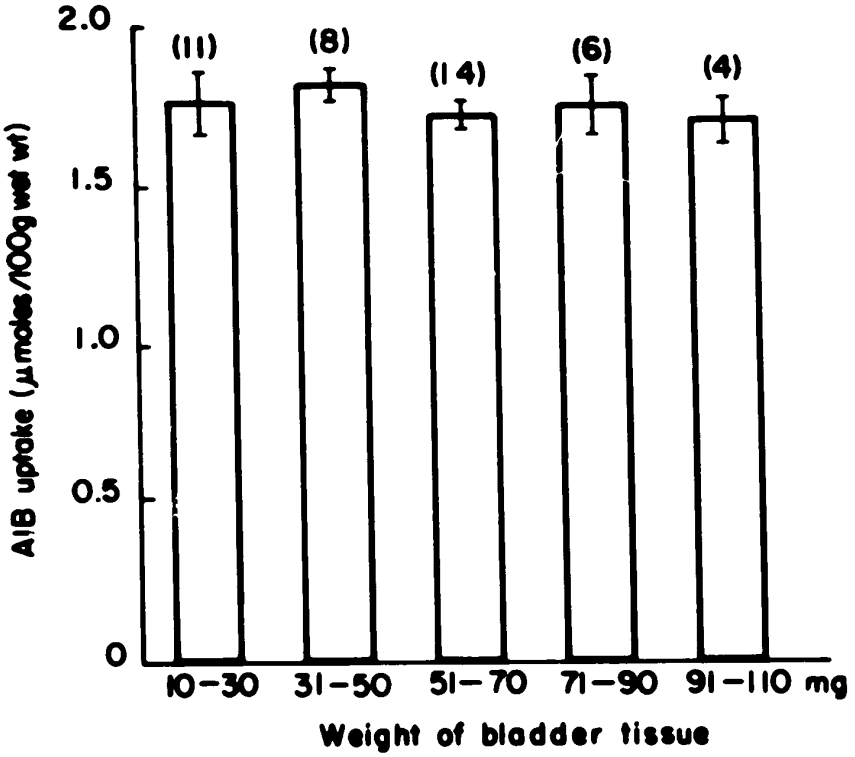


Figure 12. Effect of tissue size on α-aminoisobutyric acid uptake by rabbit detrusor muscle. Muscle strips were incubated at 37°C for 2 hr in Krebs solution containing 10μM α-aminoisobutyric acid. Each bar represents the mean of the number of observations indicated in brackets. The vertical line represents 2 standard errors of the mean.

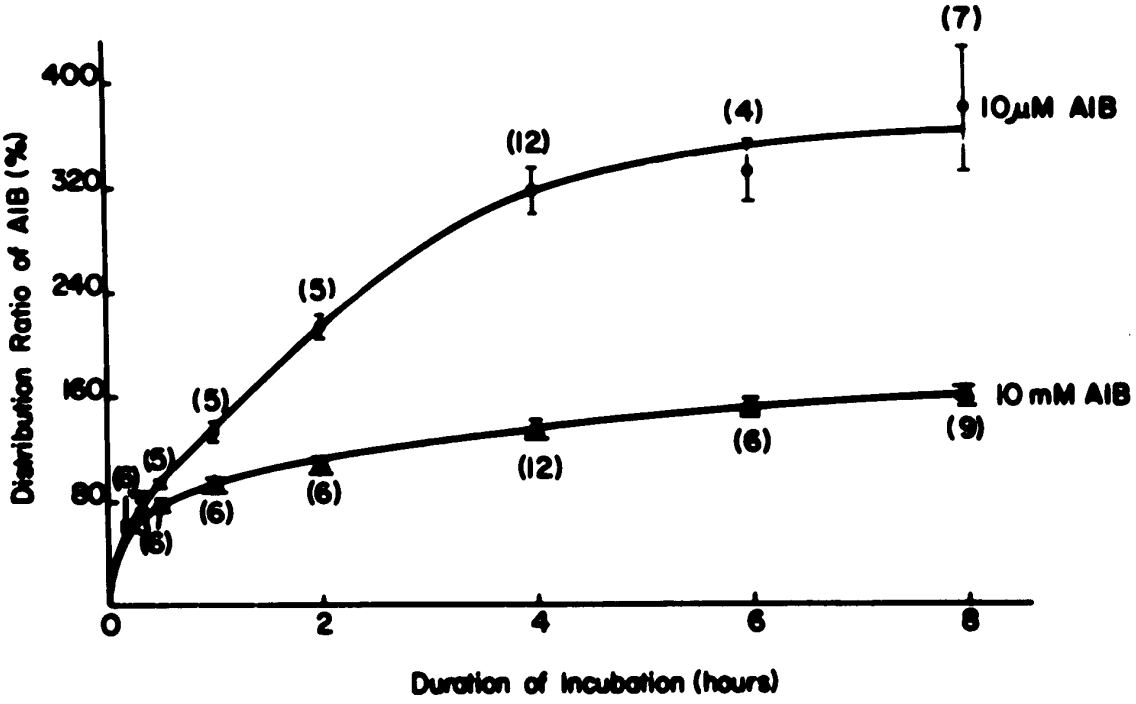


Figure 13. Effect of time on α-aminoisobutyric acid uptake by rabbit detrusor muscle. Tissues were incubated at 37°C in Krebs solution containing 10 μM (●) or 10 mM (▲) α-aminoisobutyric acid. Mean values ± S.E. Number of observations indicated in brackets. The two curves differ significantly ($p<0.05$) except after 10 min.

smaller after the first 10 minutes than that of 10 μM, and a saturation level was attained after 6 hours at a distribution ratio of only about 160%. At both 10 μM and 10 mM a distribution ratio of 60% was attained after 10 minutes. This may mainly reflect rapid equilibration of α-aminoisobutyric acid with the extracellular space and if so, would suggest an extracellular space for α-aminoisobutyric acid of about 50 ml/100 gm wet weight.

Effect of Concentration on α-aminoisobutyric Acid Uptake

The uptake of α-aminoisobutyric acid as a function of increased external concentrations of the amino acid up to 200 mM was studied. The osmotic strength of all solutions was kept constant by the addition of sucrose. Tissues were incubated for 8 hours with 0.1 mM to 200 mM α-aminoisobutyric acid and the uptake of amino acid then determined. Figure 14 shows that as the concentration was increased, the net uptake of the amino acid (expressed as μmoles/gm wet weight) increased progressively with no indication of a maximum being approached. However when the uptake is expressed as distribution ratios, it can be seen that the ratio decreased from a value of approximately 360% at 0.1mM to 100%, at 200 mM, the highest rate of decrease being below 2 mM. Redrawing the initial part of the graph on a large scale (insert of Figure 14), showed that the uptake at very low concentrations is nearly saturable. A consideration of the distribution ratios achieved shows that at 0.1 mM the ratio was 360%; at 0.2 mM, 300%; at 1 mM, 270%; and at 2 mM 200%. These findings indicate that the transport of α-aminoisobutyric acid is saturable at low concentrations

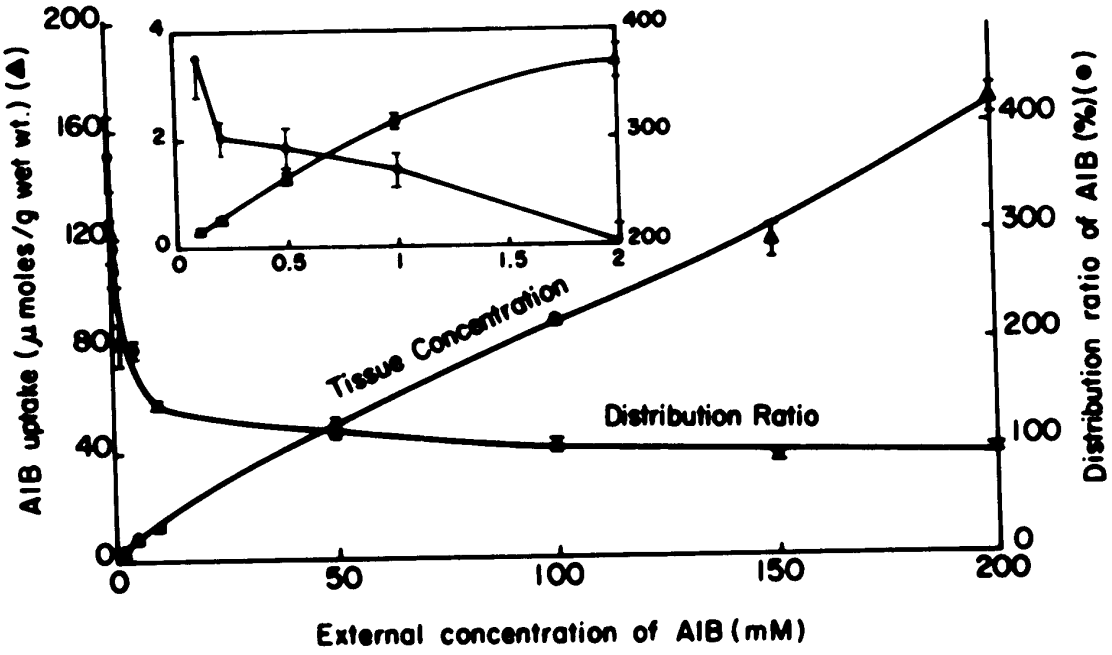


Figure 14. Effect of concentration on α-aminoisobutyric acid uptake by rabbit detrusor muscle. Tissues were incubated for 8 hr at 37°C in Krebs solution containing 100 μM - 200 mM α-aminoisobutyric acid, isoosmolarity being maintained by the addition of sucrose Mean values ± S.E. Number of observations was 5 - 9 at each point. Plotted as α-aminoisobutyric acid uptake (▲) or distribution ratio (●). Insert shows uptake and distribution ratio at 100 μM - 2 mM α-aminoisobutyric acid.

of the amino acid, and further suggest that uptake in detrusor muscle is a complex phenomenon that could involve at least two components, a transport mechanism that exhibits saturation kinetics and a linear process rather similar to passive diffusion. It should be mentioned however that the linear uptake with higher external concentrations does not necessarily mean that it is non-saturable, since the system may saturate at inaccessibly high concentrations of the amino acid.

Kinetics of α-aminoisobutyric acid uptake

As the uptake of α-aminoisobutyric acid seemed to be composed of two components, a saturable process at low external concentrations and a non-saturable process at high external concentrations, the kinetics of these two transport systems were further investigated. In these kinetic studies the initial rate of uptake of α-aminoisobutyric acid will be used and therefore it is essential to determine the time period during which the uptake of the amino acid is linear. When 0.5 mM of α-aminoisobutyric acid was present in the external medium, it was found that its intracellular uptake was linear during the 90 minutes period of the experiment (Figure 15). Thus the kinetics of uptake of 0.5 mM or less α-aminoisobutyric acid were determined after 60 minutes incubation in Krebs medium. Since the uptake of the amino acid had shown a tendency towards saturation as the concentration of the amino acid was increased (see insert of Figure 14), it seemed likely that uptake could be described in terms of Michaelis Menten equation for enzyme kinetics

$$v = \frac{Vmax\ S}{Km + S}$$

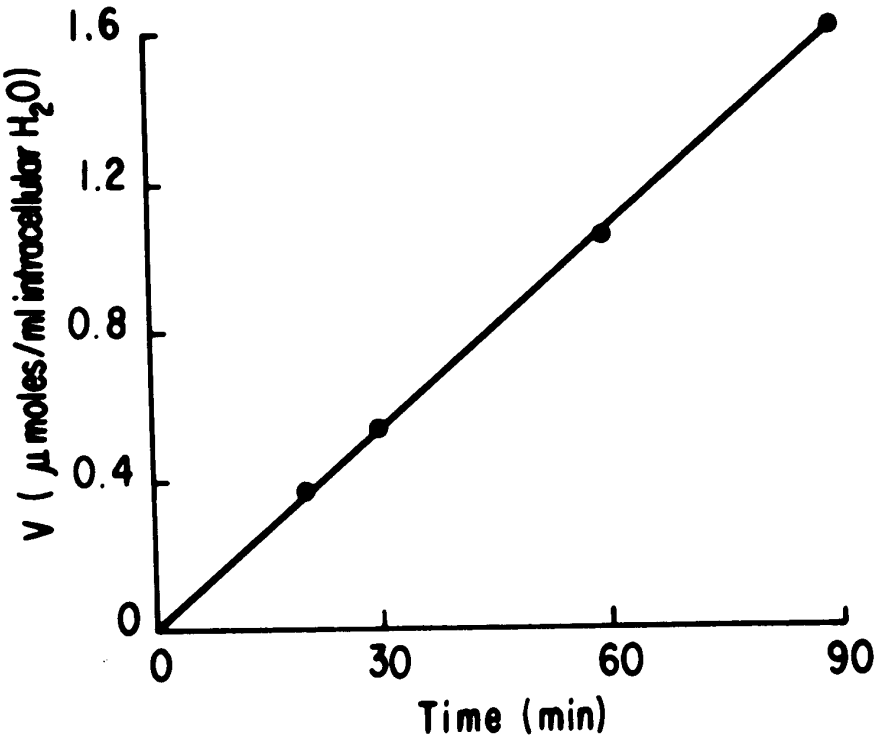


Figure 15. Initial rate of uptake of 0.5 mM α-aminoisobutyric acid by rabbit detrusor muscle. Tissues were incubated at 37°C in Krebs solution containing 0.5 mM α-aminoisobutyric acid for up to 90 min. Each point is the mean of 5 observations.

in which v is the initial velocity of uptake, Vmax is the maximal flux, S is the substrate concentration and Km is the "apparent Michaelis constant". The reciprocal of the transport rate (1/ ·) was plotted against the reciprocal of the substrate concentration (1/S) in the same way as in the Lineweaver Burk plot used for investigating enzyme kinetics (Figure 16). The straight line relationship obtained indicates a saturable rate limiting step in the process of transport. From the plot of 1/v against 1/S, the apparent Km and Vmax were determined and at the low concentrations of α-aminoisobutyric acid used, apparent Km = 0.73 mM and apparent Vmax = 1.8 μmoles/ml intracellular water per hour.

Before investigating the kinetics of the second uptake process operating at high concentrations of the amino acid the time course of uptake of 200 mM α-aminoisobutyric acid was studied to determine the time interval during which uptake was linear. Figure 17 shows that the intracellular uptake of 200 mM of the amino acid was linear during the first 20 min. only. The kinetics of uptake of the second process was studied therefore following exposure of the tissues to concentrations of the amino acid from 25 to 200 mM for 10 min. As shown in Figure 18, the straight line relationship obtained with the double reciprocal plot is an indication that uptake can be described by Michaelis Menten kinetics. The values of the apparent Km and Vmax so determined were 310 mM and 286 μmoles/ml intracellular water /10 min. respectively.

Temperature dependence of α-aminoisobutyric acid uptake

The effe.t of temperature on the uptake of 10 μM α-aminoisobu-

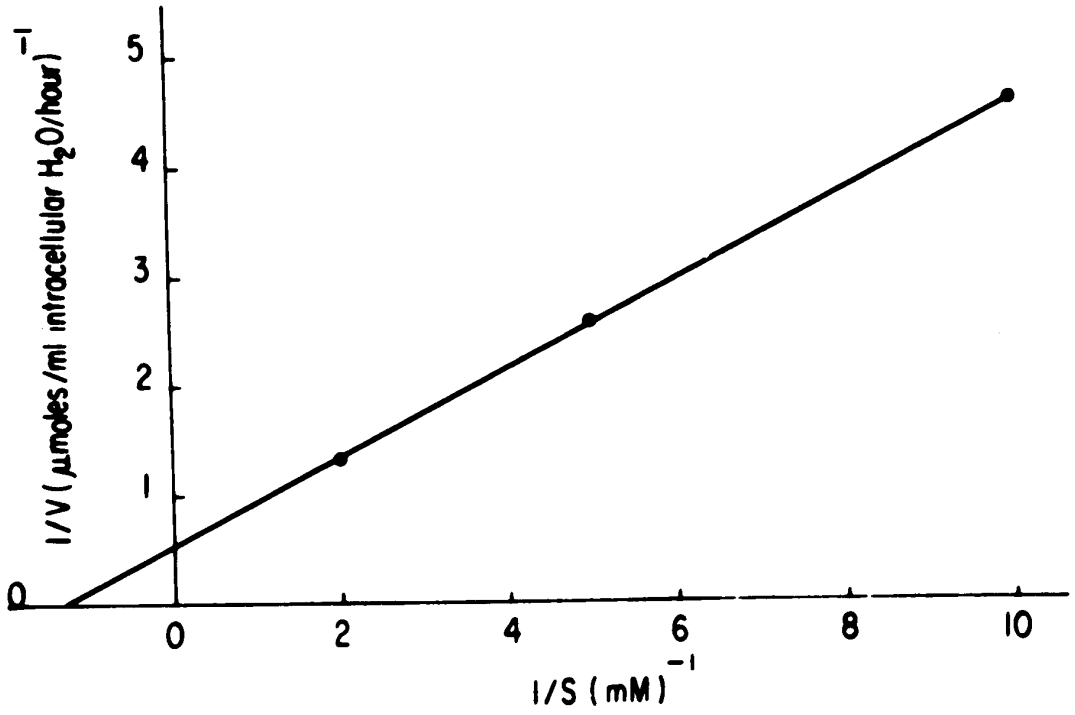


Figure 16. Determination of K_m and V_{max} for α-aminoisobutyric acid transport by rabbit detrusor muscle. Tissues were incubated for 60 min. at 37°C in Krebs solution containing 0.1 - 0.5 mM α-aminoisobutyric acid uptake. 1/V is the reciprocal of the net intracellular α-aminoisobutyric acid uptake. 1/S is the reciprocal of the external α-aminoisobutyric acid concentration. Each point is the mean of 8 - 12 observations.

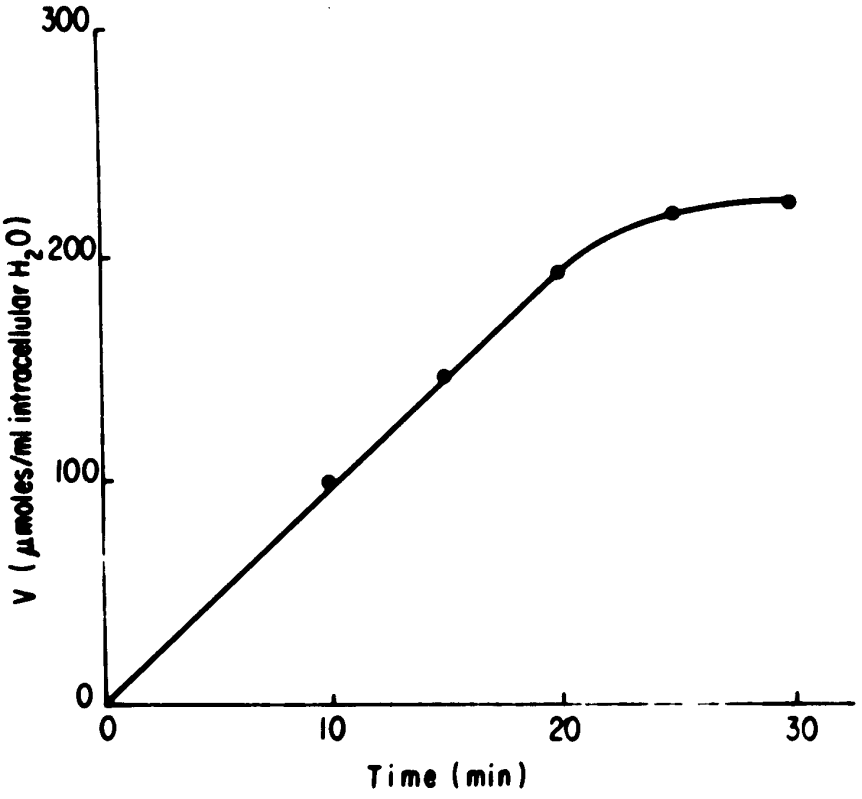


Figure 17. Determination of initial rate of uptake of 200 mM α-aminoisobutyric acid by rabbit detrusor muscle. Tissues were incubated at 37°C in Krebs solution containing 200 mM α-aminoisobutyric acid for up to 30 min. Each point is the mean of 5 observations.

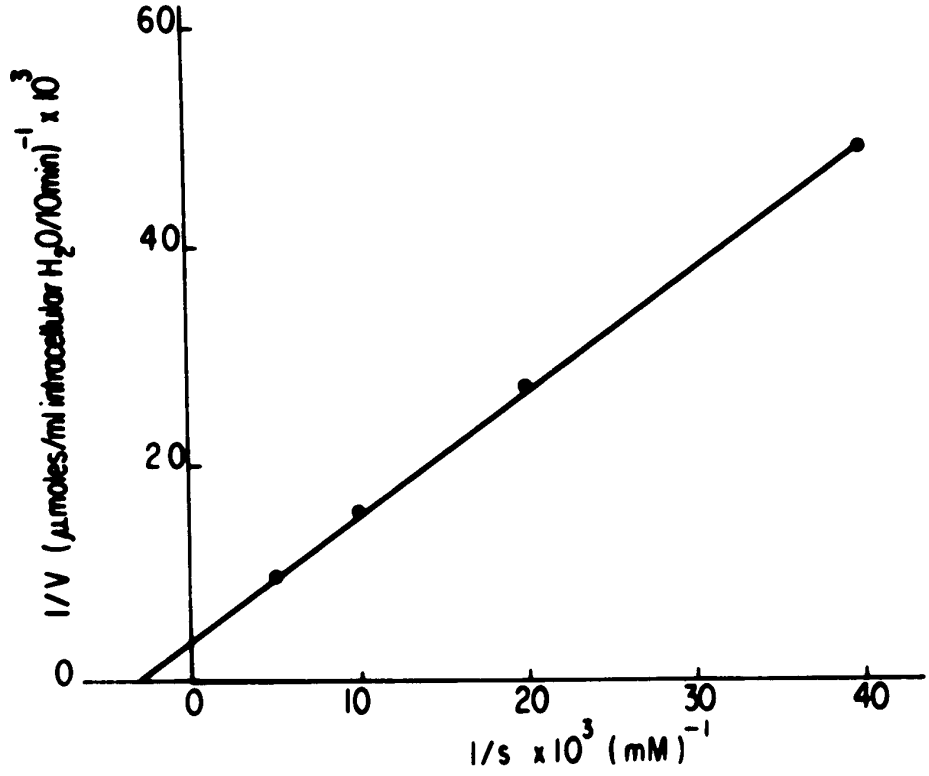


Figure 18. Determination of K_m and V_{max} for α-aminoisobutyric acid transport by rabbit detrusor muscle. Tissues were incubated for 10 min. at 37°C in Krebs solution containing 25 - 200 mM α-aminoisobutyric acid. Isoosmolarity was maintained by addition of sucrose. Each point is the mean of 6 observations.

tyric acid was determined after various periods of incubation in Krebs medium at 37°C and 27°C. Figure 19 shows that the uptake was increased by the 10°C increase in temperature with a temperature coefficient (Q_{10}) of 1.8, 2.2, and 2.1 at 1, 2 and 4 hours respectively (with an average value of 2.0). The Q_{10} was calculated after allowance was made for amino acid present extracellularly.

The observation that the uptake of α-aminoisobutyric acid was slow and that nearly steady state conditions were reached after relatively longer periods led us to investigate the energy of activation of the carrier-substrate translocation. In order to do this, the changes in Vmax with temperature were determined. Increasing the temperature from 12°C to 37°C produced a progressive increase in apparent Vmax from 0.4 to 1.8 μmoles/ml intracellular water/hour (Figure 20).

From the values of Vmax at two different temperatures, the energy of activation of the reaction can be determined from the equation

$$\log \frac{V''max}{V'max} = \frac{E\ (T_2 - T_1)}{4.576\ T_1\ T_2}$$

where V'max and V''max are the maximal velocities at the two absolute temperatures T_1 and T_2 respectively and E is the energy of activation. Taking the maximal velocities of the reaction at 12°C and 22°C as equal to 0.37 and 0.74 μmoles/ml intracellular water/hour respectively (from Figure 20) and computing for E, it was found that the energy of activation of the reaction was 11.5 Kcal/mole.

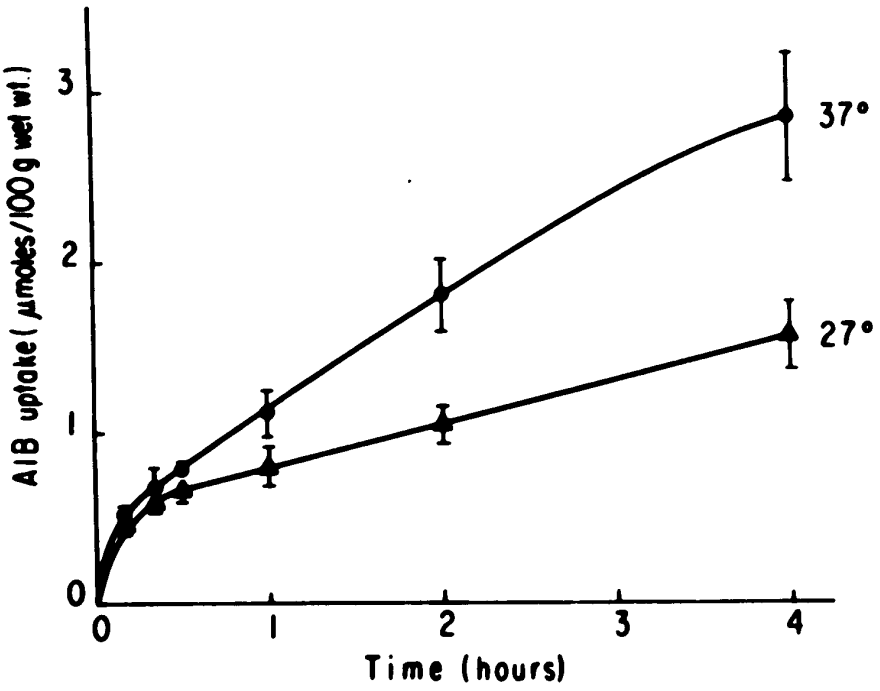


Figure 19. Effect of temperature on α-aminoisobutyric acid uptake by rabbit detrusor muscle. Tissues were incubated in Krebs solution containing 10 μM α-aminoisobutyric acid at either 37°C (●) or 27°C (▲). Mean values ± S.E. of 5 to 8 observations. The two curves differ significantly ($p < 0.05$) at all points.

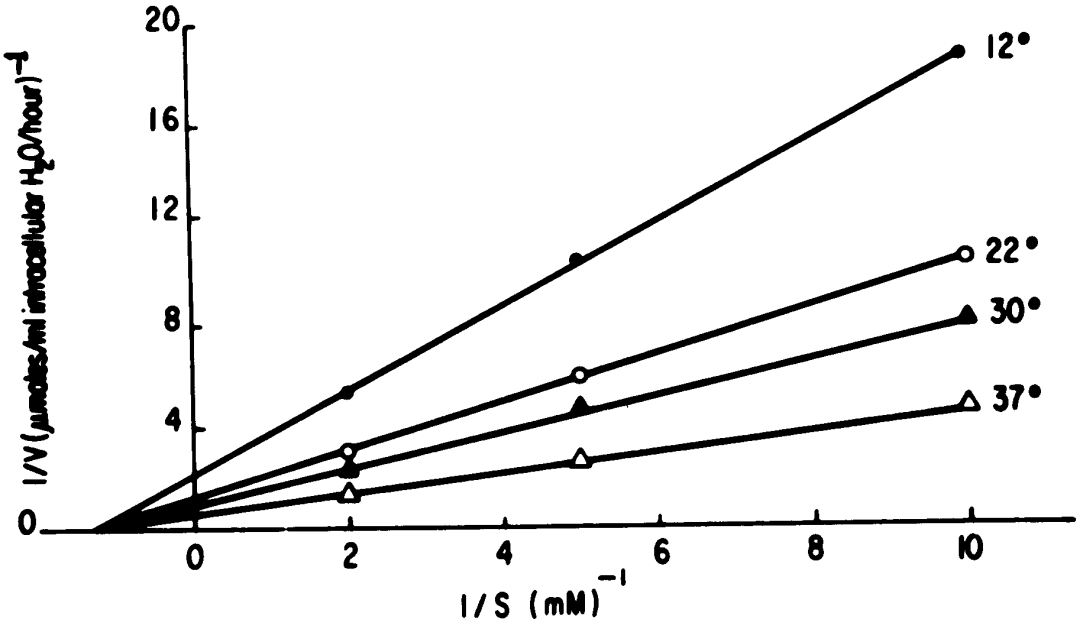


Figure 20. Effect of temperature on the kinetic parameters (K_m and V_{max}) for the transport of α-aminoisobutyric acid by rabbit detrusor muscle. Tissues were incubated for 60 min. at varying degrees of temperatures in Krebs solution containing 0.1 - 0.5 mM α-aminoisobutyric acid. Each point is the mean of 6 observations.

A more exact value of E can however be obtained by plotting log Vmax against $\frac{1}{T}$ and measuring the slope of the straight line obtained which is equal to $-\frac{E}{4.576}$. Such a plot is presented in Figure 21. By the least square fitting regression line, the slope was found to be equal to -2853 and the energy of activation thus calculated was equal to 13 Kcal/mole.

The temperature coefficient of the reaction was also determined from the ratio of the Vmax values at two temperatures. Using the maximal velocities at 12°C and 22°C, a Q_{10} of 2 was obtained, a value similar to that already obtained between 27°C and 37°C (see Figure 19). It is worth mentioning that when the Q_{10} was determined using high concentrations of α-aminoisobutyric acid (50 to 200 mM) a value of 1.2 was obtained.

Effect of Ouabain

To determine if the Na^+ and K^+ activated membrane ATPase was involved in the transport of α-aminoisobutyric acid, the effects of ouabain on transport were investigated. Tissues were preincubated in Krebs medium for one hour followed by incubation for an additional 4 hours in the same medium containing 10 μM α-aminoisobutyric acid; experimental tissues were exposed to ouabain throughout. Table XI shows the effect of ouabain on the uptake of α-aminoisobutyric acid and on total tissue water, Na^+ and K^+ contents. It is evident that ouabain produced a concentration dependent inhibition of the transport of the amino acid accompanied by a gain in tissue Na^+ and a loss of tissue K^+. 10^{-8}M ouabain had no significant effect but 10^{-5}M produced near maximal

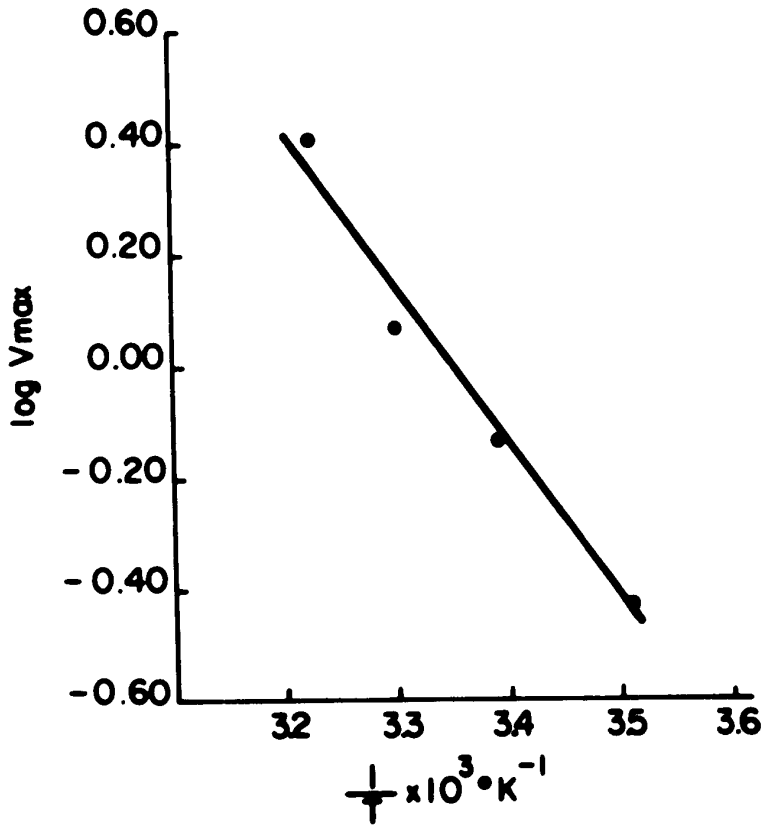


Figure 21. Arrhenius plot for the determination of the energy of activation of V_{max} for α-aminoisobutyric acid in rabbit detrusor muscle. Data for plot were obtained from Fig. 20.

TABLE XI

EFFECT OF OUABAIN ON UPTAKE OF α-AMINOISOBUTYRIC ACID BY RABBIT DETRUSOR MUSCLE

Tissues were preincubated for 60 min at 37°C in Krebs solution with or without added ouabain. Tissues were then incubated for 4 hr at 37°C in Krebs solution containing 10 μM α-amino [3-^{14}C] isobutyric acid with or without added ouabain. The values shown are the mean ± S.E. of the mean. The number of observations are shown in brackets. Values marked with an asterisk differ significantly ($p<0.05$) from values obtained in the absence of ouabain.

Ouabain (M)	Tissue H_2O content (g/100 gm)	Tissue Na^+ content (mmoles/kg wet wt)	Tissue K^+ content (mmoles/kg wet wt)	α-aminoisobutyric acid uptake (μmoles/kg wet wt)
---	86.8 ± 0.6 (6)	91.8 ± 1.8 (6)	46.0 ± 0.7 (6)	37.1 ± 3.0 (8)
10^{-8}	87.1 ± 0.8 (6)	95.0 ± 2.6 (6)	42.7 ± 2.1 (6)	31.2 ± 2.6 (10)
10^{-7}	85.1 ± 0.7 (11)	97.8 ± 2.1* (11)	35.0 ± 1.6* (11)	24.4 ± 1.4* (10)
10^{-6}	86.0 ± 0.2 (6)	106.5 ± 2.4* (6)	24.7 ± 2.6* (6)	21.1 ± 0.5* (10)
10^{-5}	87.3 ± 0.2 (6)	121.4 ± 3.4* (6)	8.9 ± 0.7* (6)	13.2 ± 0.3* (10)

inhibition of α-aminoisobutyric acid uptake and very marked downhill ion movements.

Effect of Metabolic Inhibitors, Anoxia and Glucose

The active transport of α-aminoisobutyric acid through the cell membrane of the detrusor muscle is possible only through the availability of metabolic energy produced from various metabolic processes within the cell. The major sources for ATP generation are glycolysis and oxidative phosphorylation and by the use of various inhibitors to the metabolic pathways, we could determine which pathway is contributing to the energy requirement for the transport. Whether ATP is involved directly or indirectly in the transport process will be dealt with later.

In these experiments we investigated the effects on transport of two types of metabolic inhibitors acting at two different sites, iodoacetic acid (IAA) which inhibits glycolysis through inhibition of 3-phosphoglyceraldehyde dehydrogenase enzyme (Webb, 1966) and 2,4-dinitrophenol (DNP) which is an uncoupler of oxidative phosphorylation (Slater, 1963). The results presented in Figure 22 show that the uptake of 10 μM α-aminoisobutyric acid was not significantly reduced when the tissues were preincubated for 1/2 hour in the presence of 1 mM iodoacetic acid. Transport was however slightly but significantly reduced after 90 min. exposure to the inhibitor. Following this prolonged period of preincubation with IAA, its effects however may be nonspecific i.e., inhibition of other enzymes systems may have occurred (Webb, 1966). On the other hand transport was greatly reduced by 1mM

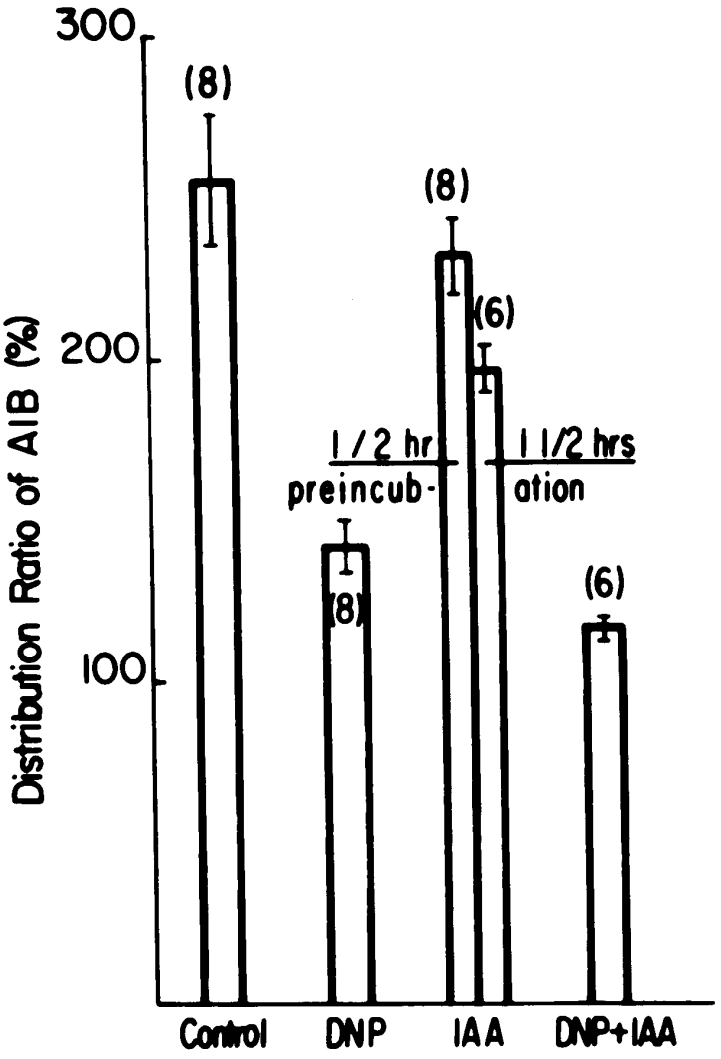


Figure 22. Effect of metabolic inhibitors on α-aminoisobutyric acid uptake by rabbit detrusor muscle. Tissues were preincubated at 37°C in Krebs solution containing 1mM dinitrophenol (for 30 min.) or 1 mM iodoacetic acid (for 30 or 90 min.) or both 1 mM dinitrophenol and iodoacetic acid(for 30 min). They were then incubated for 2 hr. at 37°C in Krebs solution containing 10 μM α-aminoisobutyric acid and 1 mM dinitrophenol, if this inhibitor had been present during preincubation. Mean values ± S.E. Number of observations indicated in brackets. All values, except that after 30 min. preincubation with 1 mM iodoacetic acid, differ significantly ($p<0.05$) from the control value obtained in Krebs solution alone.

DNP, either alone or combined with 1 mM IAA.

The lack of inhibition of the uptake following pretreatment with IAA for 30 min. led us to investigate this problem in detail since this treatment is known to prevent sodium pumping in this tissue (Munson and Paton, 1971). The time course of uptake of the amino acid was determined following 30 min. exposure to the inhibitor and as can be seen (Table XII) significant reduction of uptake starts to appear only at the end of 4 hour incubation period. However, after 2 hours there was no change in amino acid uptake and we therefore investigated the activity of the Na^+-pump at the end of this time period. The tissues were pretreated with 1 mM IAA for 30 min. and their Na^+ and K^+ contents determined after an additional 2 hours incubation in Krebs medium. It was found that the Na^+ content increased from 98 mEq/L to 117 mEq/L following treatment with iodoacetic acid while the K^+ content was reduced from 46 mEq/L to 28 mEq/L. Control tissues incubated without IAA for the same time period did not show any significant change in their Na^+ and K^+ content. Thus while the uptake of α-aminoisobutyric acid was not affected following exposure of the tissues to IAA, the activity of the Na^+-pump was markedly inhibited.

The effect of these metabolic inhibitors on the uptake of 10 mM of the amino acid was also investigated. Figure 23 shows that 1 mM DNP inhibited the active uptake by 80% of the control after allowance has been made for the passive component. The inhibition was however complete following exposure to 1 mM IAA either alone or combined with 1 mM DNP.

The effects on uptake of anoxia and exogenous glucose were also sutdied. It was found that the uptake of 10 μM of α-aminoisobutyric

TABLE XII

EFFECT OF IODOACETIC ACID ON THE UPTAKE OF α-AMINOISOBUTYRIC ACID BY RABBIT DETRUSOR MUSCLE

Tissues were preincubated at 37°C in Krebs solution in absence and presence of 1 mM iodoacetic acid (IAA) for 30 min. They were then incubated at 37°C in Krebs solution containing 10 μM α-aminoisobutyric acid for up to 4 hours. Mean values ± S.E. of 6 observations. Value marked with an asterisk differs significantly from control.

Incubation period	Uptake of α-aminoisobutyric acid (μmoles/100 gm tissue)	
	Control	IAA 10^{-3}M
10	0.52 ± 0.010	0.50 ± 0.032
20	0.69 ± 0.043	0.63 ± 0.032
30	0.78 ± 0.021	0.81 ± 0.037
60	1.12 ± 0.064	1.28 ± 0.075
120	1.81 ± 0.091	1.93 ± 0.083
240	2.85 ± 0.153	2.18* ± 0.060

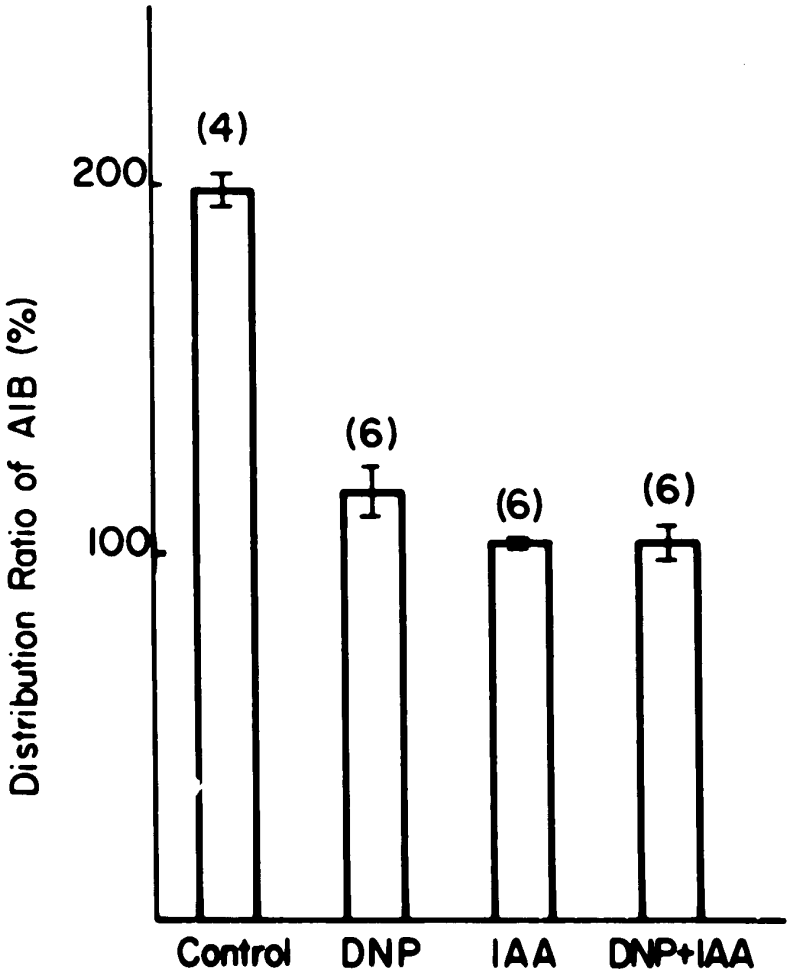


Figure 23. Effect of metabolic inhibitors on α-aminoisobutyric acid uptake by rabbit detrusor muscle. Tissues were preincubated at 37°C in Krebs solution containing 1 mM dinitrophenol (for 30 min.) or 1 mM iodoacetic acid (for 30 min.) or both 1 mM dinitrophenol and iodoacetic acid (for 30 min.). They were then incubated for 8 hr at 37°C in Krebs solution containing 10 mM α-aminoisobutyric acid and 1 mM dinitrophenol, if this inhibitor had been present during preincubation. Mean values ± S.E. Number of observations indicated in brackets.

acid was not reduced by omission of D-glucose if the medium was oxygenated (Table XIII). However the transport was greatly reduced if omission of D-glucose was combined with anoxia. If the tissues were subjected to anoxic conditions in the presence of D-glucose, uptake was slightly but significantly reduced. No change in the uptake of 10 mM AIB was observed if D-glucose was omitted from the oxygenated medium (Table XIII) or if the tissues were subjected to anoxic conditions in the presence of D-glucose. However when the tissues were incubated in glucose-free medium and under anoxic conditions, the uptake was greatly reduced (Table XIII).

Effect of Cations on Uptake of α-aminoisobutyric Acid

The effect of cations on the uptake of 10 μM α-aminoisobutyric acid was studied by incubating tissues in media in which various cations were completely replaced by iso-osmolar amounts of sucrose. In these experiments the cations were omitted both from the preincubation and incubation media. This treatment enhances the effects of the removal of the cations on the uptake of α-aminoisobutyric acid. It can be seen (Figure 24) that in absence of Na^+ there was a marked reduction in transport and since a distribution ratio of about 100% was obtained, the results provide evidence that the actively transported component was the one which was completely abolished while the passive component was not affected by removal of Na^+.

Replacement of K^+ produced a marked reduction of the concentrative uptake of α-aminoisobutyric acid; a distribution ratio of more than 100% indicated that some transport of the amino acid still occur-

TABLE XIII

EFFECTS OF SUBSTRATE AND ANOXIA ON UPTAKE OF α-AMINOISOBUTYRIC ACID BY RABBIT DETRUSOR MUSCLE

Tissues were preincubated for 60 min at 37°C in Krebs solution and were then incubated for 8 hr in Krebs solution containing α-amino [3-^{14}C] isobutyric acid. During both preincubation and incubation tissues were subjected to the treatments shown. Mean values ± S.E. of 9 - 12 observations. Values marked with an asterisk differ significantly ($p<0.05$) from values obtained in the presence of D-glucose and 95% O_2.

Krebs Solution Containing substrate (50 mM)	Krebs Solution Equilibrated with	α-aminoisobutyric acid Distribution Ratio (%) 10 μM	α-aminoisobutyric acid Distribution Ratio (%) 10mM
D-Glucose	95% O_2 - 5% CO_2	428 ± 22	132 ± 3.6
Sucrose	95% O_2 - 5% CO_2	436 ± 27	131 ± 4.4
D-Glucose	95% N_2 - 5% CO_2	316 ± 19*	131 ± 5.6
Sucrose	95% N_2 - 5% CO_2	109 ± 2*	94* ± 1.6

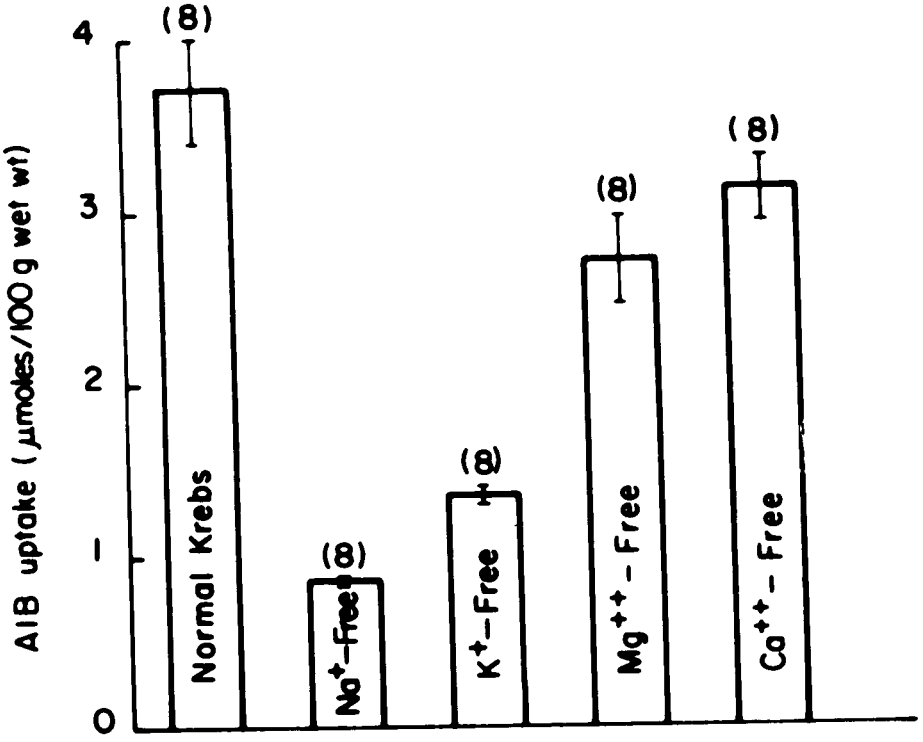


Figure 24. Effect of cations on α-aminoisobutyric acid uptake by rabbit detrusor muscle. Tissues were preincubated for 60 min. at 37°C in Krebs solution or Na^+-free, or K^+-free or Ca^{2+}-free, or Mg^{2+}-free Krebs solution. 10 μM α-aminoisobutyric acid was then added and incubation continued for 4 hr. Mean values ± S.E. Number of observations indicated in brackets. All values, except that in Ca^{2+} free solution, differ significantly ($p < 0.05$) from that obtained in normal Krebs solution.

red in absence of K^+. The percentage reduction of the active uptake due to lack of K^+ amounted to 83% of the control. It should be noted that when K^+ was omitted from the incubation media, tissues were transferred to a series of K^+ free media for 10 min. each to ensure as complete removal of K^+ as possible.

Omission of Mg^{++} produced a slight but significant inhibition of transport while omission of Ca^{++} produced no significant change in amino acid transport. These results indicate that the active uptake of α-aminoisobutyric acid is dependent on external cations; Na^+ and K^+ being essential for uptake and Mg^{++} to a lesser extent.

Since the active transport of α-aminoisobutyric acid showed an absolute requirement for Na^+, the ability of other cations (Li^+, K^+, Rb^+ and Cs^+) to substitute for Na^+ was studied. These cations were chosen because they occupy the same period in the periodic table as Na^+ denoting that they all share the same chemical properties. The tissues were preincubated for one hour in the test media followed by 4 hour incubation in the same media to which has been added 10 μM α - aminoisobutyric acid. The results in Figure 25 indicate that none of these cations was capable of supporting the uphill transport of α - aminoisobutyric acid as the distribution ratio fell from 350% in the presence of Na^+ to around 100% in its absence. The active uptake of α-aminoisobutyric acid thus exhibits a high degree of specificity for Na^+ since the inhibition of transport produced by the removal of Na^+ could not reversed either partially or completely by any one of these cations.

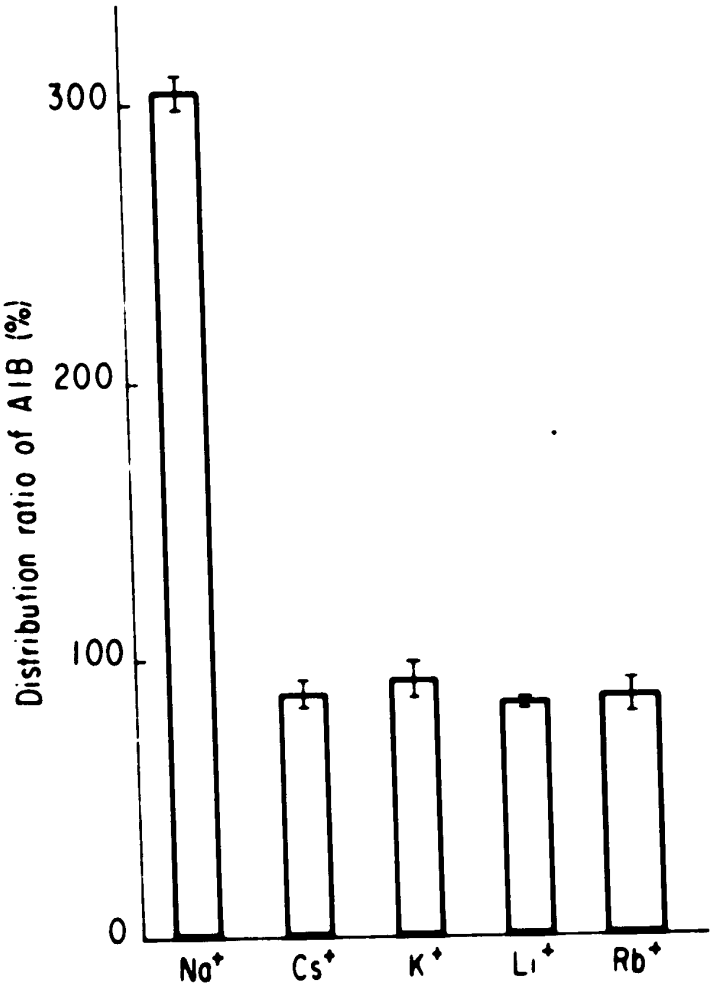


Figure 25. Effect of medium Na^+ replacement by other monovalent cations on the uptake of α-aminoisobutyric acid by rabbit detrusor muscle. Tissues were preincubated for 60 min. at 37°C in Krebs solution or Na-free Krebs solution in which Na^+ was substituted by Cs^+, K^+, Li^+ or Rb^+. 10 μM aminoisobutyric acid were then added and incubation continued for 4 hours. Each bar represents the mean of 4 determinations. The vertical line represents 2 standard errors of the mean.

Effect of Varying Medium Na^+ Concentrations

Since the ability of the detrusor muscle cells to concentrate α - aminoisobutyric acid was markedly dependent on the presence of Na^+ in the external medium, the effect of different Na^+ concentrations on the distribution ratio of the amino acid was determined. The medium Na^+ concentration was altered by iso-osmolar substitution of sucrose for Na^+ in the Krebs medium. Figure 26 shows that with 10 μM of α-aminoisobutyric acid in the external medium, the distribution ratio increased from 100% in absence of Na^+ to about 200% in the presence of 140 mM Na^+. Therefore at least under these conditions, the Na^+ dependent process appeared necessary for the transport of α-aminoisobutyric acid into the cells against its concentration gradient.

Effect of Varying Medium K^+ Concentration

Figure 27 demonstrates the changes in net uptake of α-aminoisobutyric acid when the K^+ concentration in the Krebs medium was varied from 0 to 20 mM. It can be seen that when 10 μM of the amino acid were present in the incubation medium, a fairly narrow range of medium K^+ concentration was necessary for optimal transport. Optimal transport of the amino acid occurred in the vicinity of 8 mM K^+ and was reduced at higher and lower concentrations of K^+. The uptake in the presence of 20 mM external K^+ was nearly the same as that in K^+-free medium.

Reversal of Effects of Na^+ Deprivation on α-aminoisobutyric Acid Uptake

In order to obtain further information on the uptake of α-aminoisobutyric acid by detrusor muscle, a series of experiments were carried out to examine the relation between the uptake and concen-

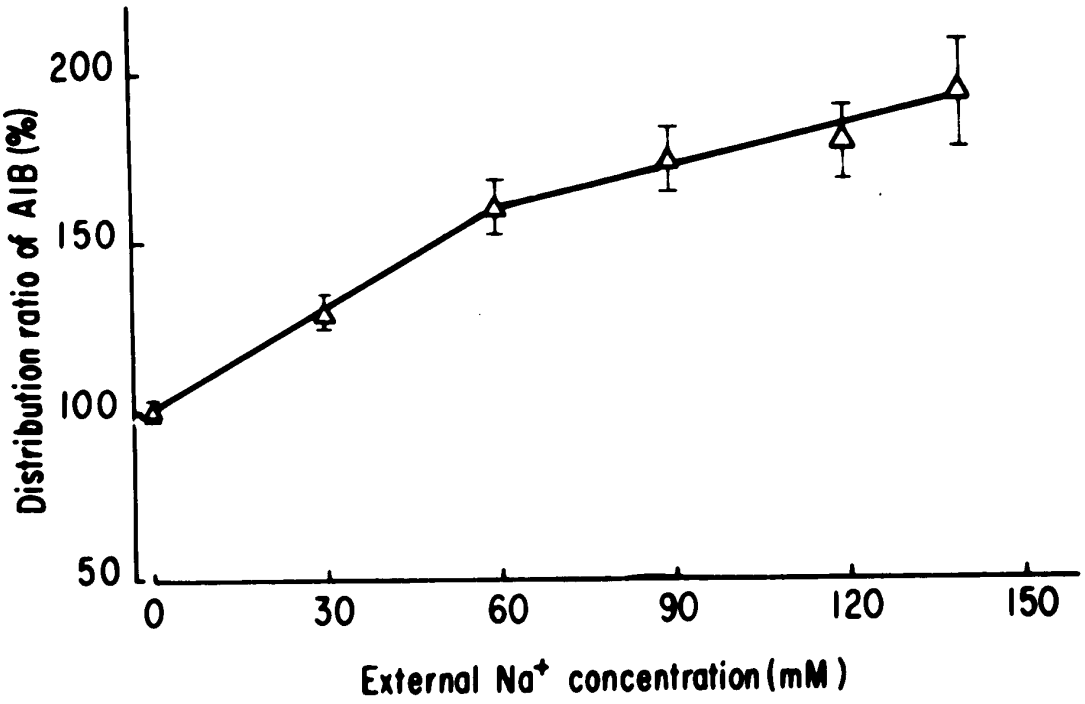


Figure 26. Effect of Na^+ on α-aminoisobutyric acid uptake by rabbit detrusor muscle. Tissues were preincubated in Krebs solution at 37°C for 60 min. at various Na^+ concentrations without αaminoisobutyric acid, then incubated for 2 hr in the presence of 10 μM of α-aminoisobutyric acid. Each point mean of 8 values ± S.E.

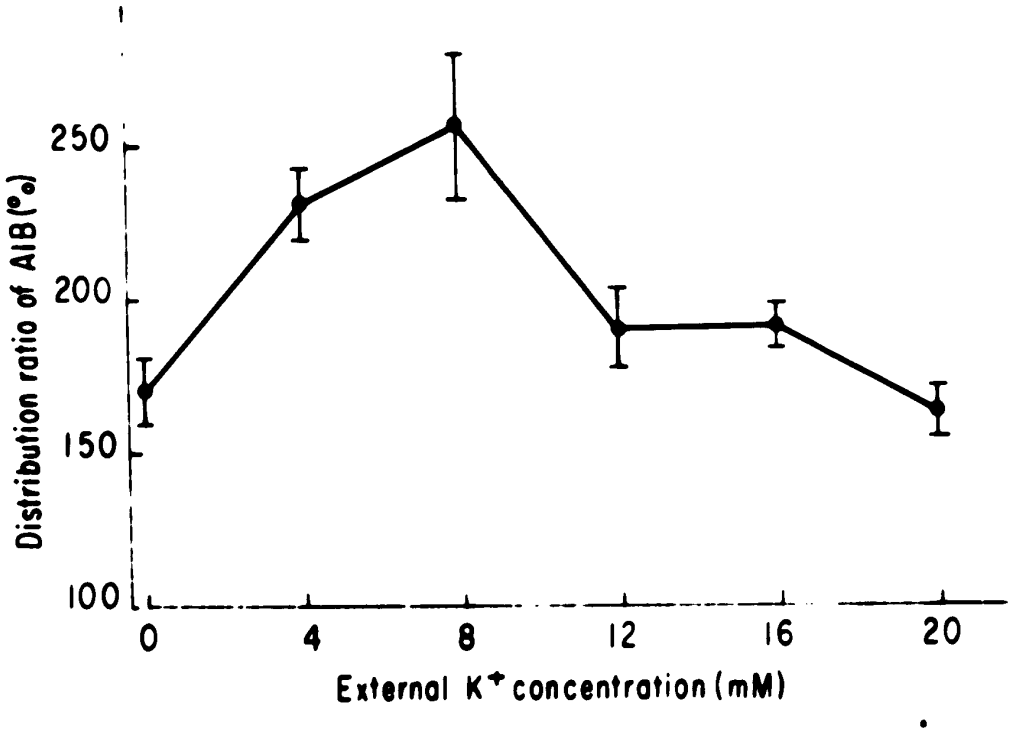


Figure 27. Effect of K^+ on α-aminoisobutyric acid uptake by rabbit detrusor muscle. Tissues were preincubated in Krebs solution at 37°C for 60 min. at various K^+ concentrations without α-aminoisobutyric acid, then incubated for 2 hr in presence of 10 μM of α-aminoisobutyric acid. Each point mean of 6 values ± S.E.

tration of the amino acid at a variety of Na^+ concentrations. Since in these experiments Na-free solution will be used, therefore it seems necessary that the substituent used for Na^+ in the Krebs Ringer medium should not produce any damage or injury to the cells, otherwise the interpretation of the results would not be correct. Some of our experiments have shown that tissues preincubated in Na-free medium with sucrose added to substitute for Na^+ did not recover completely their ability to transport the amino acid. For this reason we investigated the effects of various substituents of Na^+ on the uptake of α-aminoisobutyric acid at the end of one hour incubation period (Table XIV). It is evident from the table that although sucrose and mannitol produced a marked reduction in total tissue water, no significant effect was noticed when Li^+, NH_4^+ and $Choline^+$ were used to substitute for Na^+. The results also show that substitution of Na^+ with NH_4^+ produced significant swelling of the tissue and it may also interfere with the amino acid uptake possibly through its combination with the anionic site to which attachment of the amino group of the amino acid takes place. $Choline^+$ and Li^+ seem to be reasonable substitutes for Na^+.

To investigate the reversibility of the effects of lack of Na^+ on the uptake of α-aminoisobutyric acid, tissues were preincubated for one hour in Na-free medium in which Na^+ was replaced by iso-osmolar amounts of choline chloride. The tissues were then incubated for one hour in the same medium to which was added 10 μM α-aminoisobutyric acid; at the end of this period half of the tissues remained in the same medium while the other half transferred to a normal Krebs Ringer

TABLE XIV

EFFECT OF VARIOUS Na^+ SUBSTITUENTS ON TISSUE WEIGHT TOTAL TISSUE WATER AND UPTAKE OF α-AMINOISOBUTYRIC ACID BY RABBIT DETRUSOR MUSCLE

Tissues were preincubated for 60 min at 37°C in Krebs solution whose Na^+ content was replaced by isoosmolar amounts of Li^+, NH_4^+, choline$^+$, mannitol or sucrose. 10 μM α-aminoisobutyric acid was then added and incubations continued for 60 min. Mean values ± S.E. of 6 - 10 observations. Values marked with an asterisk differ significantly from values obtained in presence of Na^+.

Material Substituting for Na^+	Final Wt. / Initial Wt. x 100	Total Tissue H_2O (ml/100 gm wet wt.)	AIB uptake (μM/100 gm wet tissue x 10^4)
—	98 ± 1.2	85.5 ± 0.7	92.0 ± 4.5
Li^+	96 ± 1.2	86.1 ± 0.6	62.2* ± 1.5
NH_4^+	104* ± 1.7	86.3 ± 0.6	53.2* ± 1.0
Choline	94* ± 1.2	84.7 ± 0.7	57.7* ± 1.2
Mannitol	88* ± 1.7	78.5* ± 0.9	62.3* ± 2.6
Sucrose	92* ± 1.8	77.6* ± 0.6	60.8* ± 2.1

medium containing the same amount of the amino acid for further three hours. Control experiments were performed simultaneously in which the tissues were exposed to 140 mM Na^+ from the start of the experimental procedure. Figure 28 shows that when the tissues were incubated in Na^+-free medium and then transferred to normal Krebs medium, complete recovery of their concentrating ability was observed. These experiments indicate that exposure to Na^+-free choline substituted medium did not depress irreversibly the subsequent ability of the muscle cells to transport α-aminoisobutyric acid. Therefore in the subsequent experiments when Na^+ was omitted from the incubation medium, choline will be used as the substituted cation.

Effect of Na^+ on Kinetics of α-aminoisobutyric Acid Transport

The kinetics of α-aminoisobutyric acid uptake were determined in the absence and in the presence of varying Na^+ concentrations in the medium. In each experiment the uptake of α-aminoisobutyric acid was measured at 3 concentrations of the amino acid, 0.1, 0.2 and 0.5 mM using a single Na^+ concentration. Figure 29 shows that at all Na^+ concentrations tested, the uptake of α-aminoisobutyric acid could be described by Michaelis Menten kinetics since the double reciprocal plot yielded a straight line in each case. The main effect of increasing the extracellular Na^+ was to increase the value of Vmax for the uptake process. At 0,50 and 140 mM of external Na^+, the Vmax values obtained were 0.60, 0.89 and 1 38 μmoles/ml intracellular water/hour respectively. The apparent Michaelis constant, Km, however, did not undergo any significant change with changes in the Na^+ concentration and a value

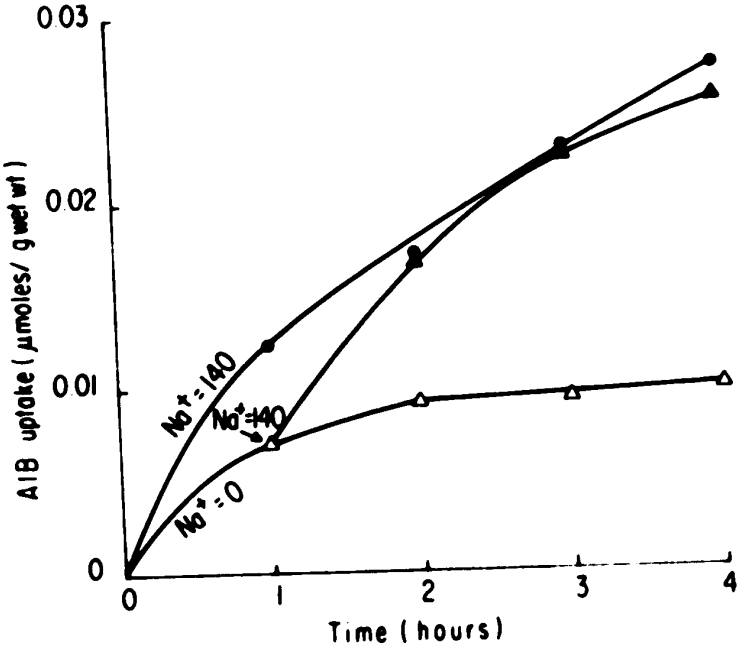


Figure 28. Reversal of effects of Na^+ deprivation on α-aminoisobutyric acid uptake by rabbit detrusor muscle. Tissues were preincubated in normal (●) or Na-free (Δ) Krebs solution at 37°C for 1 hr followed by incubation in the same medium to which is added 10 μM of α-aminoisobutyric acid for up to 4 hr. Some tissues after 1 hr incubation in the Na-free solution were then transferred to normal Krebs solution (▲), and incubations were carried out for up to 3 hr. Each point mean of 6 observations ± S.E.

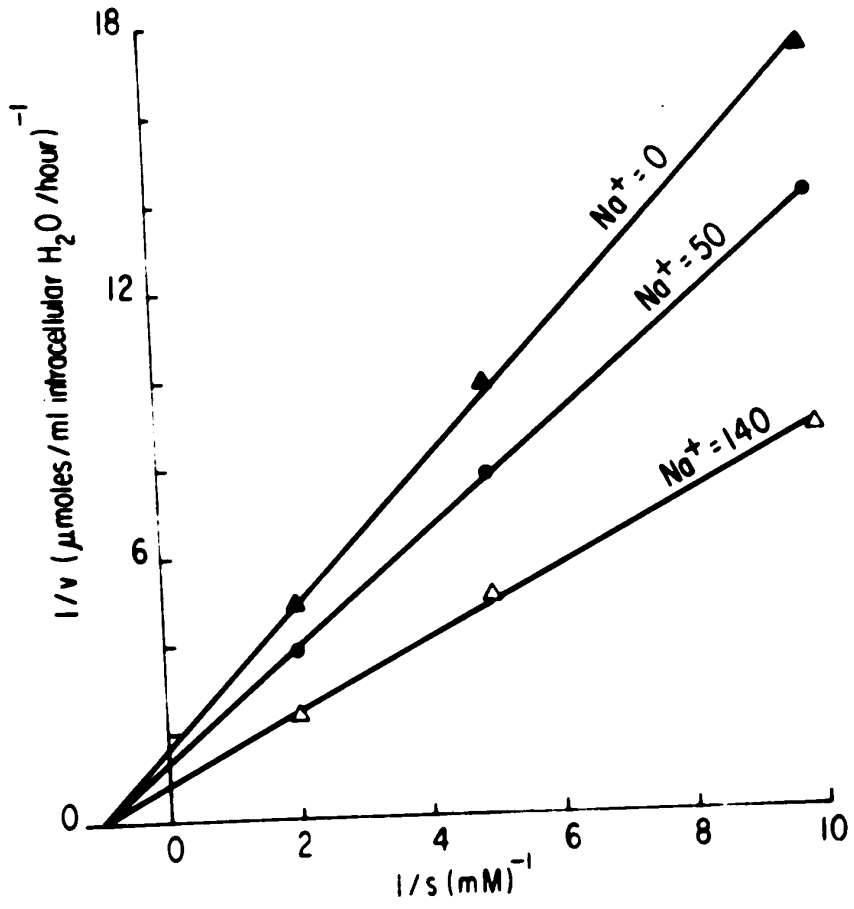


Figure 29. Effect of medium Na^+ concentration on saturation kinetics for α-aminoisobutyric acid uptake by rabbit detrusor muscle. Tissues were preincubated at 37°C for 60 min. in Krebs media containing 0,50 or 150 mM Na^+. Isoosmolarity was maintained by addition of choline chloride. Tissues were then incubated for 60 min. in the same media to which was added 10 μM α-aminoisobutyric acid. Each point is the mean of 15 observations.

near one was obtained.

The observed tendency towards saturation of α-aminoisobutyric acid uptake as a function of its external concentration in the absence of Na^+ suggests that even under these conditions, the entry of the amino acid into the cells proceeds via a mediated process. This point was further investigated by examining the effect of L-alanine on the uptake of α-aminoisobutyric acid in the absence of Na^+. As shown in Figure 30, L-alanine inhibited significantly the uptake of α-aminoisobutyric acid. Furthermore when the two stereoisomers of alanine and methionine were studied for their effects on α-aminoisobutyric acid uptake (Table XV), it was found that with alanine, the L-isomer produced more inhibition than did the D-form. With methionine however, the L-form inhibited the uptake of α-aminoisobutyric acid while the D-form produced no significant change. These results show that the transport system of α-aminoisobutyric acid exhibits a stereochemical specificity and is consistent with the concept of mediated entry in the absence of Na.

Role of Na^+ Gradient on α-aminoisobutyric Acid Uptake

The involvement of Na^+ in the active accumulation of amino acids within the detrusor muscle cells could be explained on the grounds of two hypotheses describing the accumulation of sugars and amino acids into tissue cells; either: (1) Na^+ binds to the same transport system that binds the amino acid and the Na^+ concentration difference between the two sides of the membrane provides the driving force for amino acid accumulation, or, (2) Na^+ is required for the direct input of

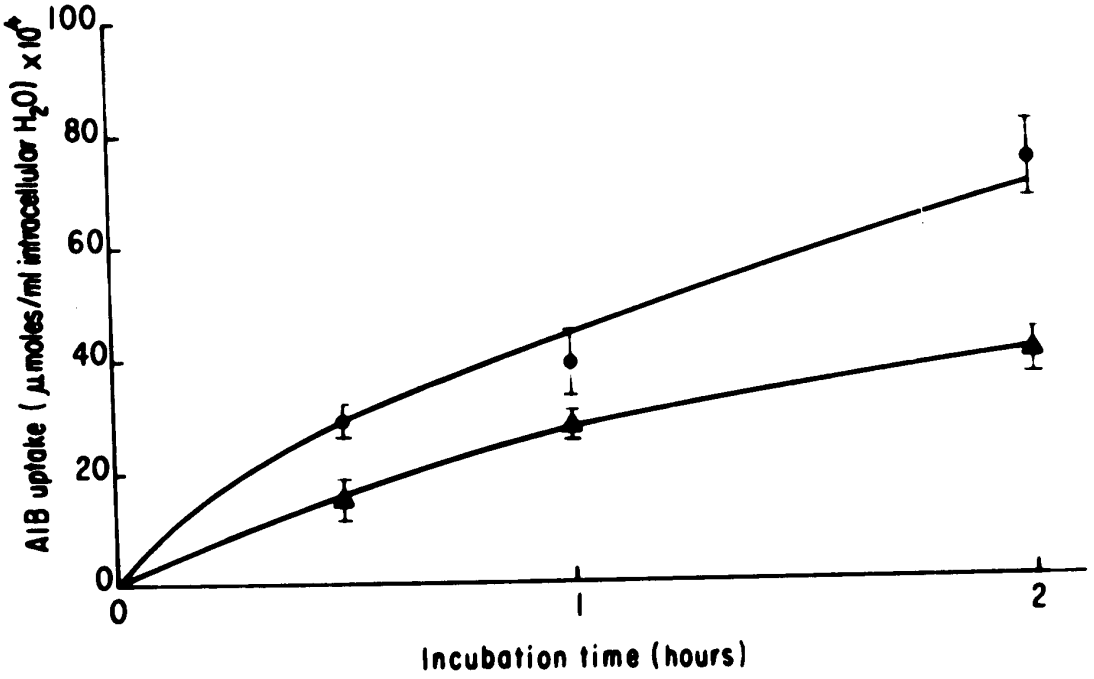


Figure 30. Effect of L-α-alanine on uptake of α-aminoisobutyric acid by rabbit detrusor muscle in absence of medium Na^+. Tissues were preincubated at 37°C for 60 min. in Krebs solution in which Na^+ salts were completely replaced by isoosmolar amounts of choline chloride, then incubated in medium containing 10 μM α-aminoisobutyric acid for up to 2 hr in presence (▲) or absence (●) of 5 mM L-α-alanine. Each point is the mean ± S.E. of 8 observations.

TABLE XV

EFFECT OF METHIONINE AND ALANINE STEREOISOMERS ON α-AMINOISOBUTYRIC ACID UPTAKE BY DETRUSOR MUSCLE IN ABSENCE OF EXTERNAL Na^+

Tissues were preincubated for 60 min at 37°C in Krebs solution whose Na^+ content was replaced by equivalent amounts of choline chloride. 10 μM α-Aminoisobutyric acid plus 5 mM of the amino acid were then added and incubations continued for 2 hr. Mean values ± S.E. of 12 observations. Values marked with an asterisk differ significantly from the control.

Amino Acid Added (5mM)	α-aminoisobutyric acid uptake (μmoles/gm tissue) x 104
—	68.0 ± 2.37
L-alanine	54.2* ± 0.92
D-alanine	62.1* ± 1.18
L-Methionine	57.8* ± 1.12
D-Methionine	63.5 ± 1.81

metabolic energy necessary for the transport possibly from the activity of the membrane Na^+/K^+ activated ATPase.

To test these two possibilities, the uptake of α-aminoisobutyric was investigated in conditions in which the Na^+ gradient across the cell membrane was changed. Accordingly, the Na^+-gradient would be able to drive α-aminoisobutyric acid transport even if the membrane adenosine triphosphatase was rendered non-functional if (1) is true but would not be able to if (2) is true.

In these experiments the tissues were preincubated in Krebs medium containing 10^{-4} M ouabain and 10 μM α-aminoisobutyric acid; this concentration of ouabain by inhibiting the Na-pump rendered the tissues Na^+ rich. Some tissues were then removed at the end of preincubation for the determination of Na^+ and amino acid content while the rest of the tissues were divided into two halves and transferred to the incubation media. One half was incubated in Krebs medium containing 140 mM Na^+ while the other half was incubated in a medium containing 30 mM Na^+. 10^{-4} M ouabain and 10μM α-aminoisobutyric acid were present throughout incubation. Tissues were removed at varying intervals for the determination of Na^+ and amino acid content. The results of this experiment (Figure 31) show that during the first 30 min. of incubation in medium containing 30 mM Na^+, despite the fact that the Na^+ gradient was directed out of the cell, the tissue content of α - aminoisobutyric acid was not different from that in the absence of any Na^+ gradient. This suggests that that the downhill movement of Na^+ could not drive the uphill movement of the amino acid but instead it may indicate that Na^+ might be required for ATPase activity and energy

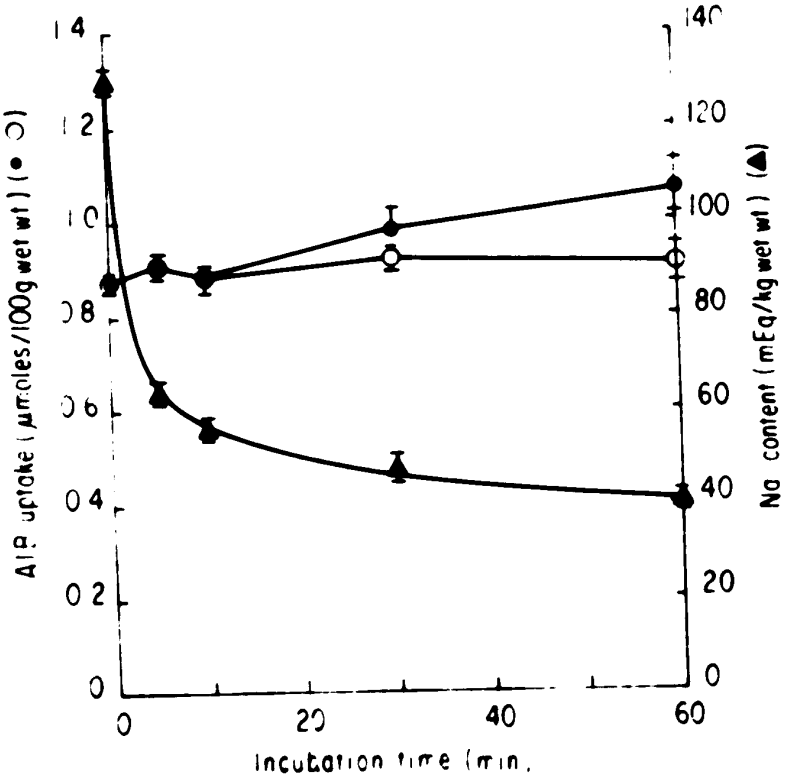


Figure 31. Effect of Na^+-gradient on the movement of α-aminoisobutyric acid in rabbit detrusor muscle. Tissues were preincubated at 37°C for 60 min. in Krebs solution containing 10^{-4}M ouabain; 10 μM α-aminoisobutyric acid was then added to the medium and tissues left for a further 60 min. • amino acid content in tissues incubated in Krebs solution, o amino acid and ▲ Na^+ content in tissues incubated in Krebs medium containing 30 mM Na^+. Ouabain 10^{-4}M and 10 μM α-aminoisobutyric acid were present in all the incubation media and incubations were continued for up to 60 min. Each point is the mean ± S.E. of 6 observations.

supply.

Inhibition of α-aminoisobutyric Acid Uptake by Amino Acids

In order to study possible competition between various amino acids and α-aminoisobutyric acid for the transport site of the latter, tissues were incubated in the presence of 10 μM α-aminoisobutyric acid with or without the addition of 1 mM unlabeled L-amino acid and the intracellular content of α-aminoisobutyric acid measured after 2 hours. Figure 32 shows that inhibition of α-aminoisobutyric acid transport was produced mainly by the monoamino-monocarboxylic acids while L-alanine produced the greatest inhibition, about 53%. To investigate the mechanism of inhibitory action of L-alanine, tissues were incubated in the presence of fixed concentrations of α-aminoisobutyric acid and different concentrations of L-alanine. It was observed that when 1 mM of alanine was used, the degree of inhibition of α-aminoisobutyric acid uptake produced was decreased as its concentration was increased. Increasing the concentration of alanine resulted in an increase of the degree of inhibition of fixed concentration of α-aminoisobutyric acid. These results suggest that L-alanine acts as a competitive inhibitor of α-aminoisobutyric acid transport. Figure 33 presents the lineweaver-Burk plot of the observed data and as is shown the results fell on straight lines which have the same y-intercept but different x-intercepts. This denotes that alanine affects the entry of α-aminoisobutyric acid by changing its affinity for the carrier while the maximum velocity is not changed.

It is also apparent that when high concentrations of L-alanine were used, the degree of inhibition was less than would have been ex-

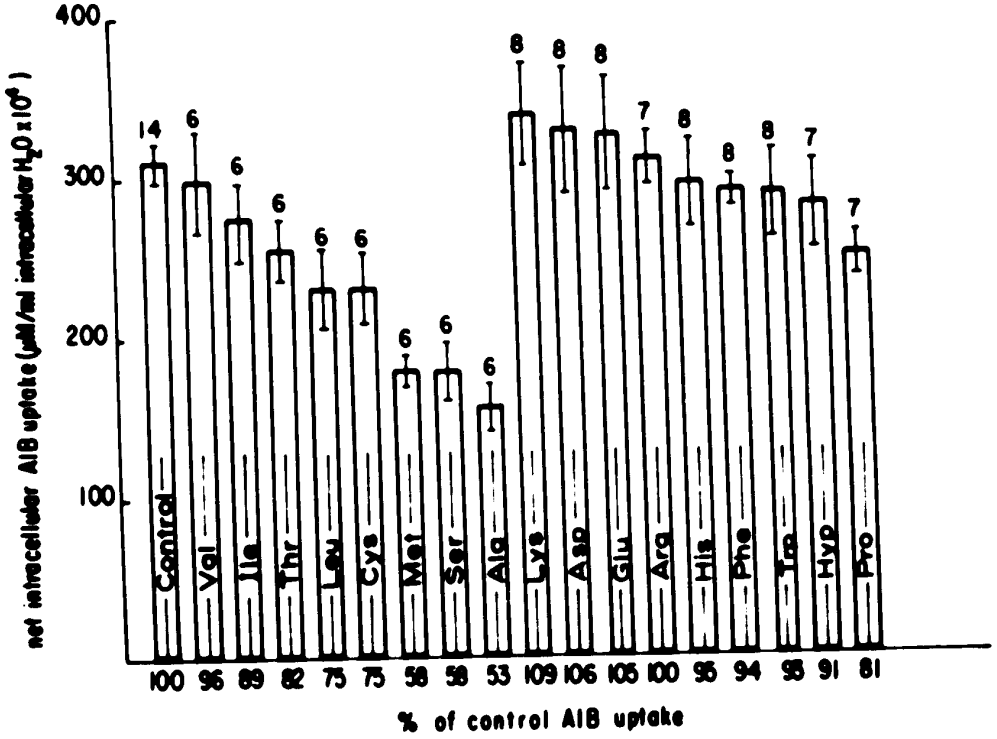


Figure 32. Effect of various L-amino acids on uptake of α-aminoisobutyric acid by rabbit detrusor muscle. Tissues were incubated at 37°C in normal Krebs solution in which the test amino acids were present at a concentration of 1 mM and the concentration of α-aminoisobutyric acid was 10 μM. Each bar represents the mean of 6 - 8 determinations. The vertical line represents 2 standard errors of the mean.

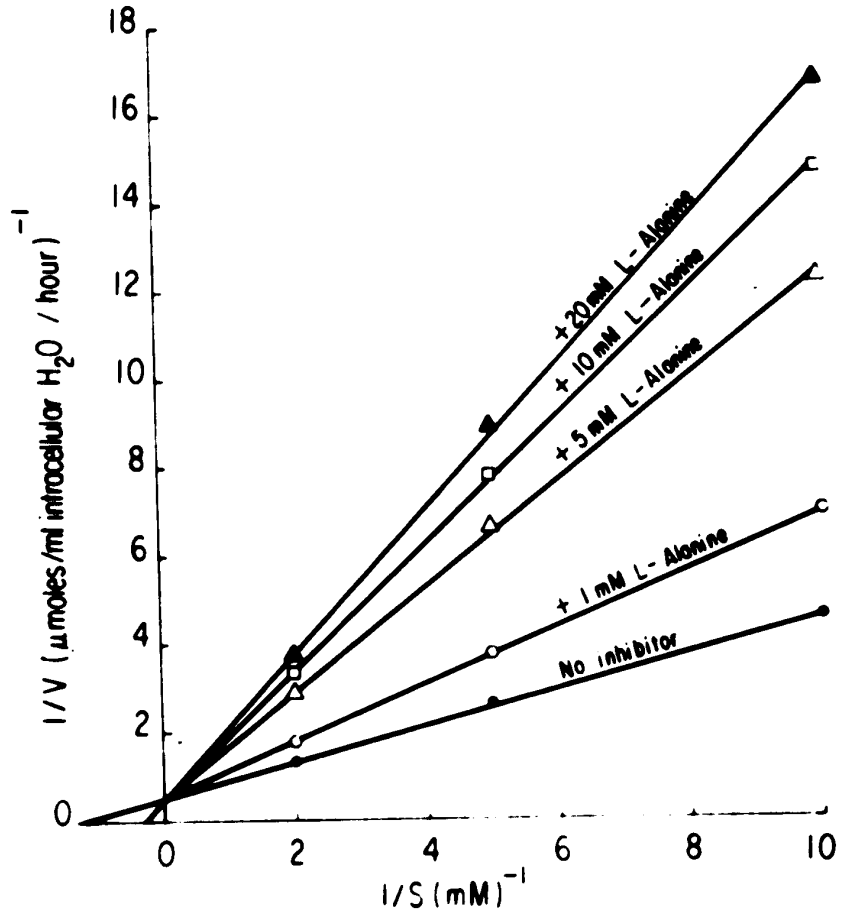


Figure 33. Double reciprocal plot to investigate the mechanism of inhibitory action of L-alanine on the transport of α-aminoisobutyric acid by rabbit detrusor muscle. Tissues were incubated for 60 min at 37°C in Krebs solution containing 0.1 - 0.5 mM α-aminoisobutyric acid and various concentrations of alanine. Each point is the mean of 7 - 12 observations.

pected from a purely competitive type of inhibitor since the inhibition produced did not increase indefinitely with an increase of inhibitor concentration. This point was studied further by plotting the reciprocal of velocity of uptake against L-alanine concentration in the presence of various concentrations of α-aminoisobutyric acid (see Figure 34). If L-alanine behaved as a purely competitive inhibitor, a straight line relationship should be obtained (Dixon, 1953). The deviation from a straight line obtained suggests that L-alanine acted as a partial competitive inhibitor of α-aminoisobutyric acid transport.

Structural Specificity of α-aminoisobutyric Acid Transport Site

The stereochemical specificity of the transport site was examined by comparing the inhibitory effects of 1 mM D-and L-amino acids on the uptake of α-aminoisobutyric acid. Table XVI shows that the L-forms of alanine, methionine, leucine and norleucine were effective inhibitors of the uptake whereas the D-forms did not exert any inhibitory effect on transport. Although D-serine produced a significant inhibitory effect, this effect was significantly smaller than that produced by L-serine. The finding that the transport system is stereospecific suggests that at least 3 groups on the asymmetric α-carbon of the amino acid must interact with the carrier (Lin et al., 1962). To identify these 3 groups a number of compounds with modifications at different substituent groups on the α-carbon were studied for their effects on α - aminoisobutyric acid uptake. It should be mentioned, however, that due to the limited availability of derivatives, the effect of modifying the carboxylic and amino groups was investigated using mainly analogues of glycine.

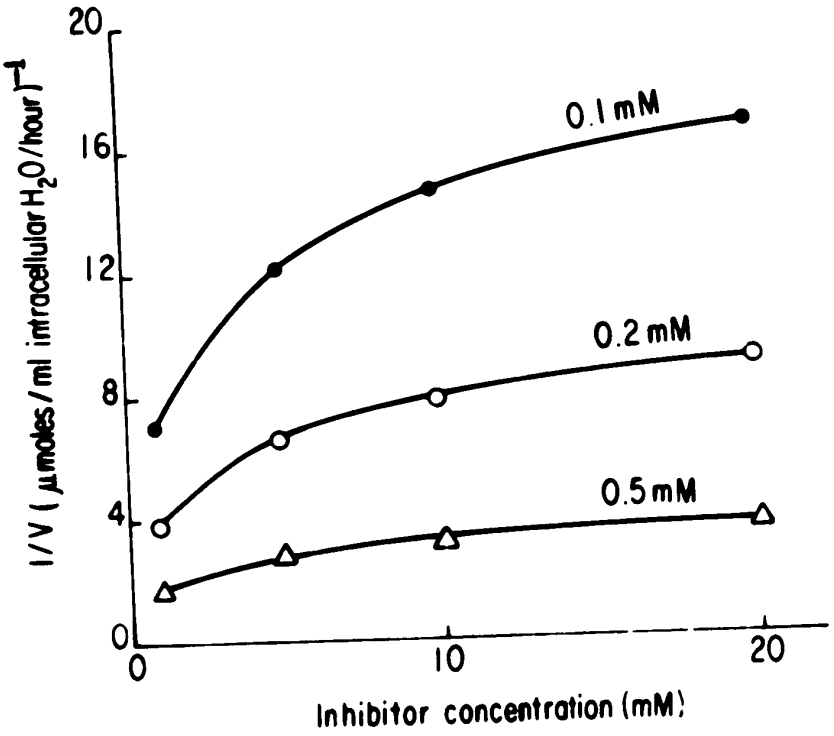


Figure 34. Plot of the reciprocal of the net intracellular α-aminoisobutyric acid against a series of inhibitor (L-alanine) concentrations keeping the concentration of α-aminoisobutyric acid constant (Dixon plot). Data for this plot were obtained from Fig. 33.

TABLE XVI

EFFECT OF STEROISOMERISM ON TRANSPORT OF α-AMINOISOBUTYRIC ACID BY RABBIT DETRUSOR MUSCLE

Tissues were preincubated for 60 min at 37°C in Krebs solution and were then incubated for 2 hr in Krebs solution containing 10 μM α-aminoisobutyric acid plus 1 mM of the amino acid. Mean values ± S.E. of 6 observations. Values marked with an asterisk differ significantly from the control.

Amino Acid (1 mM)	Intracellular AIB content (μM)	Inhibition (%)
---	30.7 ± 1.7	—
L-alanine	16.2* ± 1.7	47
D-alanine	32.9 ± 2.4	+7
L-Methionine	17.8* ± 1.3	42
D-Methionine	37.9* ± 2.4	+24
L-leucine	23.1* ± 2.6	25
D-leucine	27.8 ± 2.4	+9
L-Norleucine	21.5* ± 1.1	30
D-Norleucine	32.5 ± 2.8	+6
L-Serine	17.8* ± 2.1	42
D-Serine	23.1* ± 1.4	25

The possible importance of a free α-carboxyl group for the attachment with the transport site was investigated using two compounds, glycine ethyl ester and glycinamide, in which the carboxyl moiety has been replaced by other uncharged groups (Table XVII). In comparison with glycine which inhibited the transport of α-aminoisobutyric acid by 25%, these two compounds produced no significant inhibition. It might be argued that esterification of the carboxyl group, in addition to removing the charge, increases also the size of the substituent, hence steric hindrance cannot be excluded. However, with glycinamide the size of - $CONH_2$ group is approximately the same as that of - COOH group of glycine, yet with glycinamide there is no inhibition of the uptake of α-aminoisobutyric acid.

For the binding of the amino acid to the transport site we studied the role played by the α-amino group by using compounds with different alterations of the amino group (Table XVIII). Removal of the amino group as in β-hydroxybutyric acid produced a compound with no affinity for the carrier. This result should be compared with the 18% inhibition produced by L-threonine, a compound which has the same structure as β-hydroxybutyric acid but has in addition amino group at the α-carbon atom. Investigating the effects of some N-substituted glycines it was found that only sarcosine (N-methyl glycine) produced the same degree of inhibition of α-aminoisobutyric acid transport as did glycine while dimethyl glycine and betaine (trimethyl glycine) produced no significant inhibitory effects (Table XVIII). Furthermore glycyl glycine, N-acetyl glycine and phthaloyl glycine produced no inhibitory effects when compared with glycine.

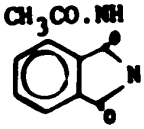
TABLE XVII

EFFECT OF CARBOXYL GROUP ON THE TRANSPORT OF 10 μM α-AMINOISOBUTYRIC ACID BY RABBIT DETRUSOR MUSCLE. INCUBATION CONDITIONS WERE AS DESCRIBED IN TABLE XVI.

Amino Acid (1 mM)		Intracellular AIB content (μM)	Inhibition (%)
	——	34.3 ± 1.5	—
$CH_2.COOH$ | NH_2	Glycine	25.8* ± 1.4	25
$CH_2.CONH_2$ | NH_2	Glycinamide	35.9 ± 2.0	+5
$CH_2.COOC_2H_5$ | NH_2	Glycine ethylester	34.6 ± 2.0	+1

TABLE XVIII

EFFECT OF AMINO GROUP ON THE TRANSPORT OF 10 μM α-AMINOISOBUTYRIC ACID BY RABBIT DETRUSOR MUSCLE. INCUBATION CONDITIONS WERE AS DESCRIBED IN TABLE XVI.

Amino Acid (1 mM)		Intracellular AIB content (μM)	Inhibition (%)
——		34.3 ± 1.5	—
$CH_3.CH(OH).CH(NH_2).COOH$	Threonine	28.5* ± 1.9	17
$CH_3.CH(OH).CH_2.COOH$	β-hydroxybutyric	38.8 ± 2.8	+13
$CH_2(NH_2).COOH$	Glycine	25.8* ± 1.4	25
$CH_2(NH(CH_3)).COOH$	Sarcosine	26.6* ± 1.9	23
$CH_2(N(CH_3)_2).COOH$	Dimethylglycine	34.7 ± 2.2	+1
$CH_2(N^+(CH_3)_3).COOH$	Betaine	33.4 ± 1.9	3
$CH_3CONH.CH_2.COOH$	Glycylglycine	32.8 ± 2.7	4
$CH_3CO.NH.CH_2.COOH$	N-acetylglycine	33.4 ± 1.4	3
	Phthaloylglycine	35.5 ± 4.5	+3

These results indicate that a free amino group is not an essential requirement for the transport, but suggest that the size of the N-substituent is important: large substituents will cause steric hindrance.

The optimal distance between the carboxyl and amino groups which is required for maximal uptake of the amino acid was investigated by studying the effects of α, β and γ-amino acid on the transport of α - aminoisobutyric acid. The results presented in Table XIX show that the transport of α-aminoisobutyric acid was strongly inhibited by α - alanine and α-aminobutyric acid while no significant effect was observed when β-alanine, β - and γ-aminoisobutyric acids were used. This shows that the attachment of the amino and carboxyl groups to the α - carbon of the amino acid is an obligatory requirement for the transport system.

In investigating the role of the side chain for the attachment with the transport site 4 different classes of amino acids were tested for their inhibitory effects on α-aminoisobutyric acid uptake. These include (1) amino acids with non polar side chain, (2) amino acids with polar side chain, (3) amino acids with charged groups on the side chain, and (4) amino acids with an aromatic or heterocyclic side chain. The results presented in Table XX show that among the amino acids with apolar side chain (methyl or methylene groups) alanine and α-aminoisobutyric acid have the maximum affinity for the transport site based on their inhibitory effects. Similar inhibitory effects were also produced by methionine and serine which have a polar side chain. However the presence of a charge in the side chain abolishes the affinity for the

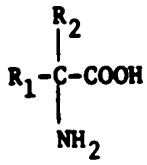
TABLE XIX

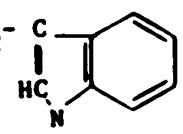
EFFECT OF α-, β AND γ-AMINO ACIDS ON TRASNPORT OF 10 μM α-AMINOISOBUTYRIC ACID BY RABBIT DETRUSOR MUSCLE. INCUBATION CONDITIONS WERE AS DESCRIBED IN TABLE XVI.

Amino Acid (1 mM)		Intracellular AIB content (μM)	Inhibition (%)
——		34.6 ± 1.5	—
$CH_3.\underset{\displaystyle NH_2}{\underset{\vert}{CH}}.COOH$	α-alanine (L)	18.1* ± 1.8	47
$\underset{\displaystyle NH_2}{\underset{\vert}{CH_2}}.CH_2.COOH$	β-alanine	33.4 ± 4.3	3
$CH_3.CH_2.\underset{\displaystyle NH_2}{\underset{\vert}{CH}}.COOH$	α-aminobutyric (DL)	26.3* ± 4.0	23
$CH_3.\underset{\displaystyle NH_2}{\underset{\vert}{CH}}.CH_2.COOH$	β-aminobutyric (DL)	39.2 ± 3.8	+14
$\underset{\displaystyle NH_2}{\underset{\vert}{CH_2}}.CH_2.CH_2.COOH$	γ-aminobutyric	34.0 ± 5.0	1

TABLE XX

EFFECT OF SIDE CHAIN ON THE TRANSPORT OF 10 μM α-AMINOISOBUTYRIC ACID BY RABBIT DETRUSOR MUSCLE. INCUBATION CONDITIONS WERE AS DESCRIBED IN TABLE XVI.



Amino Acid (1 mM) R_1	R_2		Intracellular AIB content (μM)	Inhibition (%)
—			31.4 ± 2.0	—
Apolar side chain				
- H	H	Glycine	23.9* ± 1.5	24
- CH_3	H	Alanine	16.6* ± 1.2	47
- CH_3	CH_3	α-aminoisobutyric (unlabelled)	19.5* ± 0.9	38
- $CH_2.CH_2.CH_2.CH_3$	H	Norleucine	22.0* ± 1.2	30
- $CH(CH_3)_2$	H	Valine	29.5 ± 1.6	6
- $CH_2.CH(CH_3)_2$	H	Leucine	23.6* ± 2.6	25
Polar side chain				
- $CH_2.CH_2.SCH$	H	Methionine	18.2* ± 1.4	42
- CH_2OH	H	Serine	18.2* ± 2.2	42
Charged side chain				
- CH_2COOH	H	Aspartic	32.8 ± 3.9	+6
- $CH_2.CH_2.COOH$	H	Glutamic	32.6 ± 3.6	+5
- $CH_2.CH_2.CH_2.CH_2.NH_2$	H	Lysine	34.2 ± 3.4	+9
Aromatic or heterocyclic side chain				
- CH_2 	H	Phenylalanine	29.0 ± 1.0	5
- CH_2- 	H	Tryptophan	28.8 ± 2.8	7

transport site whether the charge is positive (lysine) or negative (aspartic acid and glutamic acid). Furthermore the presence of an aromatic or heterocyclic ring in the side chain prevents the attachment of the compound to the transport site.

The results presented in Table XX show also that L-alanine has more affinity for the transport site than α-aminoisobutyric acid. Similar experiments when performed using high concentrations of these two amino acids showed similar behaviour to that already observed. Thus the α-hydrogen although not a necessary condition for the attachment of the amino acid to the transport system, yet its replacement by a methyl group reduces the affinity of the amino acid for the transport site.

V. DISCUSSION

V. DISCUSSION

A. EXTRACELLULAR SPACE

The results obtained with the detrusor muscle strips in the present investigation accentuate the difficulties involved in obtaining an unequivocal estimate of the true extracellular space in the detrusor muscle from the steady state volumes of distribution of external solutes. This study showed that different substances equilibrated in detrusor muscle with spaces of different volumes, the spaces occupied by mannitol and sucrose (about 60 ml/100 gm) being considerably larger than those occupied by inulin, light-and heavy-dextran (about 43 ml/100). Similar findings have been reported in studies of the extracellular space of guinea-pig taenia coli (Goodford and Leach, 1966), canine intestinal circular muscle (Barr and Malvin, 1965), frog smooth muscle (Bozler and Lavine, 1958) and canine carotid arterial muscle (Villamil *et al.*, 1968). By contrast in longitudinal muscle from guinea-pig ileum sucrose, inulin and mannitol equilibrated with spaces whose volumes did not differ significantly (Weiss, 1966).

The discrepancy in the size of the spaces occupied by different substances in this tissue could possibly be ascribed to the fact that very large molecules such as inulin do not occupy the whole of the extracellular space because of exclusion, possibly by mucopolysaccharides (Ogston and Phelps, 1961). This possibility was examined by studying the effects of hyaluronidase and the results reported herein for the uptake of individual markers have shown that their volumes of distribution within the muscle was decreased after the muscle had been expo-

sed to various hyaluronidase treatments. Shrinkage of the tissues was a concomitant finding while significant reduction of the Na^+ and K^+ contents of tissues was only observed after treatment with high concentration of the enzyme. The reason why treatment with hyaluronidase caused tissue shrinkage is not immediately obvious. The present results are in contrast with those observed in guinea-pig taenia coli where the inulin space increased significantly after treatment with hyaluronidase although the ionic composition and wet weights did not undergo any significant change (Goodford and Leach, 1966). With high concentration of the enzyme (50 i.u./ml), however, the latter authors observed a slight reduction in tissue K^+ which approached the 5% significance level. In canine carotid arterial muscle, Villamil et al. (1968) have stated that "The addition of large amounts of hyaluronidase to the incubating fluid had no effect on the uptake or space of inulin at different pH". However, the results presented in Figure 4 of their paper showed that at physiological pH, the inulin space was significantly reduced from 40 to 34 ml/100g wet weight after treatment with hyaluronidase; these workers did not apparently measure the weight changes during incubation. Although hyaluronidase affected the intercellular matrix, as confirmed histologically in the present study, yet there was no increase in the space occupied by these markers. This suggests that either the extracellular hyaluronic acid did not produce any steric hindrance to the penetration of these markers or shrinkage of the tissues could have negated effects on mucopolysaccharides.

The smaller molecules (i.e. mannitol and sucrose) used probably penetrated the cell membrane in view of their extremely large equilibration volumes. It seems very unlikely that as much 70% of the total tissue water can be extracellular in this tissue. Bozler and Lavine (1958) obtained evidence that sucrose penetrated the cells of frog stomach muscle. Recent work however suggests that even compounds of large molecular size can penetrate intracellularly. By means of autoradiography, Nicholls and Wolfe (1967) showed that [^{14}C]-inulin and [^{14}C]-dextran entered nerve and glial cells of Leech ganglia while Brown et al. (1969) found that [^{3}H]-inulin, [^{3}H]-mannitol and [^{35}S]-sulfate penetrated neurones of cat sympathetic ganglia. In both cases however, the degree of penetration was very small.

An analysis of the efflux of inulin, sucrose and mannitol showed that the semilog plots of tissue radioactivity against time could be described by three compartments and a bound fraction for all three compounds. It is also noteworthy that efflux was not complete after 6 hours. The efflux of sucrose in frog stomach muscle (Bozler, 1966); inulin in guinea pig taenia coli (Born, 1962), sucrose, inulin and dextran in Leech ganglia (Nicholls and Wolfe, 1967) was also shown to be multicompartmental in nature and to possess a slowly effluxing component. The anatomical localization of such compartments is at present unknown but it seems possible that some of the slowest compartments could be intracellular.

It should be mentioned that the method used for the fitting of efflux decay curves to linear exponential equations is subject to many

difficulties (Robertson, 1957; Daniel and Robinson, 1970; Goodford, 1970; Cook and Taylor, 1971). First, any error in calculating the bound fraction will lead to serious errors in the sizes of compartments and their rate constants. Second, the last exponent at large values of "t" is the one with the greatest errors due to the relatively low count rates at this portion of the curve. Because this is the starting point of resolving the curve into its original exponents, any errors tend to accumulate as the analysis proceeds. Third, application of the least square fit during each stage of the analytical procedure results in overweighting the later portion of the curve and does not guarantee a least square fit for the original curve. Fourth, it cannot resolve any two components with very close rate constants. Fifth, it does not give an error estimate, without which the evaluation of the results will be difficult. Sixth, there is an occasional difficulty in distinguishing two from three compartment systems.

The graphical method commonly employed (Perl, 1960) suffers from similar disadvantages to those mentioned above, and involves an additional error arising from the need for human judgment; this last source of error is not present in any computer assisted analysis. The mathematical procedure employed in these studies places few restrictions on the data; no estimate of the error of each point is required and the data do not need to be equally spaced with respect to time.

It should be emphasized that every method for exponential curve fitting which has been reported suffers from its own advantages and disadvantages. Thus although the method employed here is open to

criticism, its use can be justified not only for the reasons mentioned above, but also because it is simple, rapid and readily available.

The intracellular contents of Na^+ and K^+ were calculated from the total tissue water and electrolyte contents and the equilibration volumes of the various extracellular markers. By means of similar calculations Barr and Malvin (1965) showed that in canine intestine, arabinose and mannitol penetrated to greater volumes than could be extracellular since the space occupied by these compounds was larger than the Na^+ space. In the present study similar findings were obtained using [^{14}C]-sucrose. Calculation of intracellular contents of ions poses problems of interpretation however because there is evidence that Na^+ is bound to negatively charged groups located in the extracellular matrix (Palaty et al., 1969) while the Na^+ content of the fastest component of efflux exceeded that present in the apparent extracellular space in guinea-pig taenia coli and rabbit myometrium (Brading and Jones, 1969; Jones, 1970), in canine carotid artery (Jones and Karreman, 1969) and in rat uterine horns (Daniel and Robinson, 1970).

Our observations on the effect of temperature on the uptake of inulin and mannitol have shown that at early incubation periods the uptake of both markers is slow at 2°C compared with that at 37°C, but with prolonged incubation the uptake values at both temperatures approached each other. This indicates that the space available within the muscle for the distribution of inulin and mannitol is the same at 37°C or 2°C, although the diffusion process is slow at 2°C. Similar

findings have also been reported for the uptake of sucrose in longitudinal muscles from guinea-pig ileum (Weiss, 1966). In mouse brain slices, however, Cohen et al. (1968) have shown that lowering the temperature from 37°C to 0°C produced a reduction in the space occupied by inulin and since the two uptake curves at long equilibration periods were nearly parallel, they suggested that inulin is accessible to a real tissue compartment at 37°C but not at 0°C. The term "second inulin space" was thus introduced which they defined phenomenologically as the difference between the inulin space at 0°C and 37°C. Sucrose, mannitol, sorbitol and sulfate showed a similar behaviour (Cohen et al., 1970). Although the location and nature of this second marker space is not known, it was proposed that a ramifying system of tubules extends throughout the cell body which are continuous with the extracellular space; an increase in temperature increases the diameter of the tubules and subsequently the extracellular markers will penetrate more producing the phenomenological second marker space. This tubular system offers also an explanation to their observation that small molecular weight compounds produced a large volume of distribution compared with large sized molecules at both 37°C and 0°C, if the diameter of the tubules were comparable to the size of the molecules. In smooth muscles, however, this situation seems unlikely as it is well known that the sarcoplasmic reticulum which communicates freely with the extracellular space is poorly developed in most smooth muscles compared with striated muscles (Burnstock, 1970).

When the cellular barriers were disrupted by intense metabolic

inhibition (glucose free + iodoacetic acid + DNP), the spaces occupied by sucrose and mannitol were markedly increased. On the other hand the volumes of distribution of inulin, light and heavy dextran did not undergo any significant change; an indication that the cell membrane prevented their diffusion into the cells. In comparison with similar studies performed in arterial wall (Villamil et al., 1968), it was found that the inulin space was increased from 35.9 to 57.4 ml/100 gm wet weight on exposure of the tissues to Na iodoacetate plus Na cyanide. However, in renal cortex slices the space occupied by inulin was not significantly different whether the incubations were carried under aerobic or anaerobic conditions (Whittam, 1956).

The observation that the space occupied by mannitol in presence of exogenous glucose was identical to that in absence of glucose rules out the possibility that mannitol and glucose share a common transport system for their entry into the cell. That mannitol and sucrose do penetrate intracellularly is inferred from their volumes of distribution which approached the total tissue water in the presence of metabolic inhibitors.

In view of the possible cellular penetration by mannitol and sucrose with the consequent large volumes of distribution, it seems that inulin may be the best marker available at present to measure the extracellular space in this tissue. In using inulin care however should be exercised because [^{14}C]-inulin obtained from different commercial sources has been shown to provide different estimates of the extracellular space in mouse brain slices (Levi, 1969) and in rab-

bit lens (Paterson, 1968), the difference being attributable to the inulins being of different average molecular size.

B. AMINO ACID TRANSPORT

The foregoing results demonstrate that detrusor muscle strips concentrate α-aminoisobutyric acid from the incubation medium. Since α - aminoisobutyric acid is not metabolized or incorporated into proteins, it seems likely that it remains in free solution in the intracellular fluid (Goodman, 1966) although binding to some intracellular sites cannot be excluded.

A prolonged period of incubation was required for transport, to reach equilibrium as has been observed in certain other tissues (Riggs et al., 1968; Tews and Harper, 1969). However it was found that as the concentration of α-aminoisobutyric acid was increased, uptake became linear with no evidence of saturation up to 200 mM. A similar phenomenon has been observed in other tissues (Akedo and Christensen, 1962 a; Vidaver, 1964; Scriver and Mohyuddin, 1968; Touabi and Jeanrenaud, 1969). Christensen and Liang (1966) have shown that the so-called non-saturable uptake of amino acids by Ehrlich ascites tumour cells does not occur by simple diffusion since it exhibits chemical specificity, has a high Q_{10} and is markedly pH sensitive.

The kinetics of transport of α-aminoisobutyric acid was apparently amenable to analysis by the Michaelis Menten equation and the transport seems to be composed of two saturable processes, one operating at low external concentrations and the other at high ones. Matthews and Laster (1965) have discussed the interpretation of kinetic relationships and have concluded that they do not demonstrate the

the nature of the transport mechanism and are only an indication of a saturable rate-limiting step in the process of transport.

It must, however, be mentioned that saturation kinetics per se are far from characterising the process operating at high external solute concentrations and further experiments are needed to explore the properties of this process.

In kinetic studies correction of the saturable component from the linear component has been carried out by Akedo and Christensen (1962 b) and by Vidaver (1964) using two different approaches. Both authors assumed that the two processes were mediated by two distinct transport systems and were operating simultaneously. They, however, used concentrations of the amino acid in their kinetic studies which were large relative to Km and the contribution of the second process at these large amino acid concentrations might therefore be significant. In the present study, there is much less reason to make similar corrections since kinetic studies of the first process were performed at low concentrations of the amino acid (up to 0.5 mM) so that the contribution of the second process to the total uptake under these conditions may be assumed to be negligible. This assumption was based on the following observations.

1) The Km of the first process is 0.73 mM while that of the second process is 310 mM, thus it seems unlikely that the second process will be significantly affecting the net total uptake in the presence of low concentrations of the amino acid in the external medium.

2) Measurement of the intracellular uptake of α-aminoisobutyric acid at 37°C and 27°C from an external concentration of 10 μM and calculation of Q_{10} it was found to be 1.8, 2.2 and 2.1 at 1, 2 and 4 hours respectively. If the two processes were taking place simultaneously and the second process was contributing significantly to the total uptake, then with prolonged periods of incubation a reduction in Q_{10} value is to be expected. This is due to the low temperature sensitivity of the second process (Q_{10} of 1.2). It might be argued however, that these Q_{10} measurements were made with 10 μM α-aminoisobutyric acid in the external medium while in kinetic studies concentrations up to 0.5 mM of the amino acid were used. However when the temperature coefficient was calculated from the Vmax values at 12°C and 22°C, a value of 2 was also obtained. This denotes that if a significant part of the net uptake with these low concentrations of the amino acid was due to the second process, the Q_{10} should be reduced at these low temperatures.

An increased rate of transport of α-aminoisobutyric acid as well as an increase in the steady state level was obtained by an increase in the temperature from 27°C to 37°C with a temperature coefficient of 2.1. The calculated Q_{10} value at lower temperatures (between 12°C and 22°C) was nearly the same indicating that no conformational changes of the transport system occur at low temperatures. This is in contrast to the observations of Tenenhouse and Quastel (1960) where different Q_{10} values were obtained for glycine transport

in Ehrlich ascites cells at different temperatures.

The observed value for Q_{10} in our studies is similar to that observed for α-aminoisobutyric acid uptake in rat diaphragm (Kipnis and Parrish, 1965), bone cells (Rosenbusch *et al.*, 1967) and guinea pig lymph cells (Helmreich and Kipnis, 1962) although it is markedly less than that obtained for Ehrlich tumour cells (Oxander and Christensen, 1963b). It is worth mentioning that a high Q_{10} *per se* is not indicative of active transport process. Davson and Danielli (1943) have pointed out that diffusion across a thin lipid layer from one aqueous phase to another may be very slow and show a high Q_{10} so that a Q_{10} of 2 or 3 (or even higher) cannot be taken to exclude diffusion as the rate limiting step.

In the present study lowering the temperature reduced the Vmax for transport while the Km is not changed. This observation is in variance with that observed in Ehrlich ascites cells (Jacques *et al.*, 1970) where the Km for α-aminoisobutyric acid transport increased as the temperature was decreased. The activation energy for α-aminoisobutyric acid transport was found to be 13.1 Kcal/mole which is similar to the values reported by the latter workers for a number of transported amino acids. The full significance of the energy of activation can only be comprehended if the mechanism of the reaction is known; in such case its value will be attributed to the specific rate limiting step. Assuming that the translocation of the substrate carrier complex is the rate limiting step, then the energy of activation for Vmax for transport will determine the velocity of the overall reaction.

In consideration of energy requirements, the aforementioned results show that α-aminoisobutyric acid is taken up by the detrusor muscle by two distinct processes. The first process is a downhill movement where no metabolic energy is required; the asymmetric distribution of the amino acid between the two sides of the cell membrane could drive it across the membrane. The second is an uphill transport against the concentration gradient with the energy provided by the various metabolic processes inside the cell serving as the driving force. It should be emphasised that this division into a passive and an active process is an operational and not a mechanistic classification.

With low concentrations of the amino acid (10 μM) in the incubation medium, the energy for uphill transport is derived mainly from the metabolism of endogenous fats and proteins *via* the citric acid cycle as evidenced by the lack of inhibition produced by iodoacetic acid during the two hour incubation period. The presence of an exogenous energy source is not required as omission of glucose from the external medium did not affect the uptake process. The effect of glucose on the uptake of amino acids by other tissues was found to be either stimulatory (Munck, 1968 a; Abadom and Scholefield, 1962 a; Bingham *et al.*, 1966), inhibitory (Thier *et al.*, 1964) or to have no effect (Tews and Harper, 1969). The manner in which glucose affects the transport of amino acids could be due to its ability to provide an additional energy source (Newey and Smyth, 1964 b; Munck, 1968 a) although competition with amino acids for the same transport system

has been reported (Alvarado, 1966; Hindmarsh et al., 1966). The inability of glucose to affect the uptake of α-aminoisobutyric acid in our studies precludes its possible transport by the same carrier system as that which transports the amino acid.

In contrast with iodoacetic acid, 2,4-dinitrophenol markedly inhibited the uptake of α-aminoisobutyric acid which substantiates the proposal that the metabolism of endogenous fats and proteins is the major source of energy. Anaerobiasis however produced slight but significant reduction of the amino acid uptake. Assuming that the system was strictly anaerobic and oxidative phosphorylation was inhibited, then the only explanation is that the activity of anaerobic glycolytic pathway was enhanced and supplied the required energy for transport; in such case an exogenous glucose source is required. Oxidative phosphorylation however, could continue under these presumably anaerobic conditions if minute quantities of molecular oxygen were present in the vicinity of the mitochondria (Bricker and Klahr; 1966). If we ignore this last possibility, then the effects of 2,4-dinitrophenol should be the same as those of anaerobiasis except if 2,4-dinitrophenol, in addition to being an uncoupler of oxidative phosphorylation, could inhibit the uptake by some other mechanism(s). 2,4-Dinitrophenol has been shown in other tissues to stimulate the mitochondrial ATPase activity (Lardy and Wellman, 1953; Myers and Slater, 1957; Pullman et al., 1960) and consequently available ATP will be dissipated by virtue of this increased ATPase activity. Under such circumstances, 2,4-dinitrophenol is expected to have more inhi-

bitory effects on amino acid transport than does anaerobiasis alone. If these assumptions hold in our tissue then the endogenous glycogen stores could also be a secondary energy cource. This last source is important with prolonged periods of incubation (4 hours) as iodoacetic acid starts to produce an inhibitory effect. An argument however could be raised here, that there is a lag period before iodoacetic acid starts to exert its effects. As evidence against this it was shown that 30 min. exposure to iodoacetic acid did not significantly affect the uptake of the amino acid and yet the Na-pump was markedly inhibited. Similar treatment with iodoacetic acid has been shown to inhibit the Na-pump in these muscle preparations (Paton, 1969; Munson and Paton, 1971) as well as the contractility (Paton, 1968).

With high concentrations of α-aminoisobutyric acid in the external medium (10 mM) iodoacetic acid abolished the concentrative uptake denoting that the endogenous fats and proteins are no longer able to supply the energy for transport and the contribution from the endogenous glycogen stores or exogenous glucose is required. The lack of inhibition in glucose-free medium excludes the possibility of exogenous glucose as an energy source for transport. The inability of anaerobiasis to affect the uptake and the marked inhibition in the presence of 2,4-dinitrophenol could be explained on the same grounds as those mentioned when 10 μM of α-aminoisobutyric acid was present in the external medium.

From the aforementioned discussion it seems that both glycoly-

sis and respiration could supply the energy for the concentrative uptake of α-aminoisobutyric acid, an observation similar to that reported for amino acid uptake by Ehrlich ascites cells (Tenenhouse and Quastel, 1960) small intestine (Baker and Copp, 1965), uterus (Riggs et al., 1968), liver (Tews and Harper, 1969) and skeletal muscle (Bombara and Bergamini, 1968). However, in brain (Abadom and Scholefield, 1962 a) and kidney (Rosenberg et al., 1961) the uptake of amino acids was completely dependent on aerobic metabolism.

There are, however, some difficulties that should be considered in evaluating the effects of various metabolic inhibitors in whole cell or tissue preparation:

1) The concentration of inhibitor in the external medium may be different from its concentration at its site of action in the cell (Laws and Stickland, 1967).
2) The action of these inhibitors when studied in isolated systems need not be the same when studied in the more complex intact cell system.
3) If no metabolic parameters are measured, it is difficult to assign a definite site of action to these agents
4) The effective metabolic inhibitors reduce the level of ATP in the cell (Abadom and Scholefield, 1962 a; Potashner and Johnstone, 1970) but there is no direct evidence of such a reduction in the concentration of ATP in the membrane where the transport is taking place.

The studies performed to investigate the ionic requirement for

the transport have indicated that the extracellular Na^+ is unique in its ability to influence the active transport of α-aminoisobutyric acid in detrusor muscle. The inorganic monovalent cations Li^+, K^+, Rb^+ and Cs^+ when used to substitute for medium Na^+ were not able to duplicate the effect of Na^+ and could not support the transport of the amino acid against its concentration gradient. Similar studies concerning the role of extracellular Na^+ in amino acid transport in other tissues are in general agreement with these observations (Begin and Scholefield, 1964; Vidaver, 1964; Christensen et al., 1967; Schultz et al., 1967; Riggs et al., 1968; Scriver and Mohyaddin, 1968; Thier, 1968; Tews and Harper, 1969; Touabi and Jeanrenaud, 1969).

When Li^+, choline$^+$. NH_4^+, sucrose or mannitol were used as substituents for Na^+ and the uptake of α-aminoisobutyric acid monitored after one hour incubation, it was found that the use of these different Na^+ substituents resulted in different transport rates. The uptake of the amino acid in Li^+, mannitol and sucrose-substituted media did not differ significantly. However there was significant tissue shrinkage and loss of total tissue water with mannitol and sucrose substitution for Na^+. The latter two parameters did not change significantly when Li^+ was substituted for Na^+. A different picture was seen with NH_4^+ and choline substitution where the uptake of α-aminoisobutyric acid was more seriously reduced. With both substitutents the total tissue water did not change significantly, but there was significant tissue swelling with NH_4^+ compared to shrinkage with cho-

line substitution.

Our finding that the uptake of the amino acid was the least when NH_4^+ was used, and that this was accompanied by swelling of the tissue suggest its possible intracellular penetration perhaps by combining with the anionic site of α-aminoisobutyric acid transport system. Sucrose mannitol and choline chloride are not perfect substituents either. The use of sucrose and mannitol to replace external sodium is accompanied by a reduction of external chloride concentration and of the ionic strength. This will affect the membrane properties by reducing the potassium permeability (Casteels, 1970). Choline was also found to penetrate slowly into skeletal muscle fibres (Renkin, 1961), cardiac muscle fibres (Boulpaep, 1963) and reduces the K content of the guinae-pig taenia coli (Casteels, 1970). A similar effect has also been observed with Li^+ and it was found to penetrate the intestinal cells and replace K^+ (Daniel, 1965). These aforementioned problems indicate clearly that there is no ideal substituent available for Na and a proper choice between them is difficult. Data that resembled in many aspects the present results have been observed for the uptake of amino acids and sugars in a variety of tissue cells when various substituents were used for Na^+ but in addition Li^+ was found to have a stimulatory effect in some cases (q.v. Introduction).

The data presented in Table XIV shows also that omission of Na^+ from the external medium slowed α-aminoisobutyric acid penetration. Bihler et al. (1962) have shown that the rate of sugar equili-

bration by the hamster small intestine under anaerobic conditions was greater in Na^+ medium compared to a medium in which K^+ was used to substitute for Na^+. This Na^+ dependent anaerobic entrance being observed with the actively transported and not with the non-actively transported sugars led them to suggest that it is a part of aerobic active transport process and that Na^+ stimulates energy-independent as well as energy-dependent transport processes. Similar findings were also observed when the uptake of α-aminoisobutyric acid was studied in rabbit intestinal preparations (Rosenberg et al., 1965) where Na^+ deprivation reduced the rate of transport of the amino acid. In addition these workers observed that when the concentration gradient of the amino acid was abolished by metabolic inhibitors, a reduction of medium Na^+ from 144 to 25 mEq/L produced further reduction of tissue amino acid. That Na^+ affects the rate of transport of α-aminoisobutyric acid is also evident from the kinetic studies in presence of varying medium Na^+ concentrations. Na^+ in the external medium was found to change the velocity of translocation of the substrate-carrier complex and the value of Vmax increased hand in hand with increase in medium Na^+ concentration. These findings indicate that Na^+ is required for facilitating transport of α-aminoisobutyric acid even when it is moving down its concentration gradient where energy supply is not required.

The stereochemical specificity of the transport system as well as the saturation kinetics observed in absence of Na^+ indicate that the transport process under these conditions is carrier-mediated.

Some differences, however, did exist between the behaviour of the stereoisomers when Na^+ was removed from the external medium; D-alanine was inhibitory to the uptake of the amino acid in absence of Na^+ compared to the lack of inhibition in presence of Na^+. It may be possible that the attachment of the D-amino acid to the carrier was modified by the presence or absence of Na^+ (Margolis and Lajtha, 1968).

The transport of α-aminoisobutyric acid in detrusor muscle was dependent on the extracellular K^+ concentration. Omission of K^+ from the incubation medium inhibited the uptake of the amino acid to a greater extent, although some active uptake was still operating as evidenced by a distribution ratio of more than 100%. Furthermore a narrow range of medium K^+ concentrations was required for maximal transport of the amino acid and the transport was greatly reduced at lower and higher K^+ concentrations. A similar dependence of α-aminoisobutyric acid accumulation has been reported to exist in fat cells (Touabi and Jeanrenaud, 1969), renal cortex slices (Fox et al., 1964), liver cells (Tews and Harper, 1969) and right ventricular papillary muscles (Lesch et al., 1970) although removal of K was without effect on the transport process in intestinal preparations (Reiser and Christiansen, 1967).

The role played by the divalent cations in the transport phenomenon has been shown to be less prevalent than the monovalent Na^+ and K^+. The few published observations indicate that not all substances have the same degree of dependence on Ca^{2+} for transport across the cell membrane. In our studies α-aminoisobutyric acid uptake was

not significantly affected by removal of Ca^{2+} from the incubation medium; a finding similar to that already observed for the uptake of valine by the intestine (Reiser and Christiansen, 1967) and α-aminoisoburytic acid by kidney cortex (Fox et al., 1964). In liver slices, however, Ca^{2+} deprivation reduced the uptake of α-aminoisobutyric acid (Tews and Harper, 1969) whereas replacement of Ca^{2+} by Sr^{2+} increased the uptake of inositol in renal cortex slices (Hauser, 1969). Furthermore in brain slices the absence of Ca^{2+} inhibited the uptake of tyrosine (Gurroff et al., 1961), glycine (Abadom and Scholefield, 1962b)and aminoisobutyrate (Lahiri and Lajtha, 1964), increased the uptake of γ-aminoisobutyrate and glutamate (Tsukada et al., 1963) and did not affect the uptake of cycloleucine (Lahiri and Lajtha, 1964). The manner in which Ca^{2+} might influence the transport process is not clearly understood but it is well known that Ca^{2+} is involved in the maintenance of the functional integrity of the cell membrane (Manery, 1966). In addition Ca^{2+} has been shown to inhibit Na/K dependent ATPase (Rifkin, 1965; Epstein and Whittam, 1966) as well as activating the Na-pump (Fankenhaeuser and Hodgkin, 1957; Kleinzeller et al., 1968).

In absence of Mg^{2+} the uptake of α-aminoisobutyric acid was found to be slightly but significantly reduced; similar results have also been observed for amino acid uptake in the brain (Abadom and Scholefield, 1962a; Gurroff et al., 1961) and renal cortex slices (Brown and Michael, 1971).

The marked inhibition of the active uptake by the lack of Na^{+}

in the external medium, the partial inhibition by the lack of K^+ and the slight inhibition by the omission of Mg^{2+} verifies the ionic dependence of the transport to Na^+, K^+ and to a lesser extent to Mg^{2+}. Whether this dependence is due to the presence of these ions in the extracellular fluid and/or the intracellular medium is unknown.

It was observed that in the cell membrane there exists an enzyme that hydrolyzes ATP to ADP and P_i and requires Na^+, K^+ and Mg^{2+} for maximal activity (Skou, 1962); an ionic requirement that parallels those already described for α-aminoisobutyric acid uptake against its concentration gradient. This Na^+/K^+-activated ATPase have been isolated from the membrane fraction of vascular smooth muscle (Wolowyk et al., 1971) and its role in membrane transport has been thoroughly reviewed (Charnock and Opit, 1968). It requires an optimal concentrations of Na^+ and K^+ for its proper activity (Charnock and Post, 1963); Na^+ is required internally and K^+ externally. Whereas the requirement for Na^+ was found to be specific, the effects of K^+ however, could be duplicated by other monovalent cations (Skou, 1960). Inhibition of enzyme activity is expected if the tissues were incubated in K-free solution but if however some K^+ leaks from the internal to the external surface of the membrane, the energy yielding process could operate and concentrative uptake could take place. This might explain why a distribution ratio of more than 100% is observed in tissues incubated in K^+-free media.

A unique property of the Na^+/K^+-activated ATPase is its specific inhibition by ouabain, a cardiac glycoside which also inhibits

the Na^+-pump as well as the uptake of α-aminoisobutyric acid against its concentration gradient in our muscle preparations. The observation that ouabain inhibited the uptake of α-aminoisobutyric acid simultaneously with inhibition of Na^+-pump might be explained on the grounds of a common energy source that has been inhibited.

The close similarity between the properties of this Na^+/K^+-activated ATPase and the uphill movement of various electrolytes and non-electrolytes across the cell membrane might suggest that Na^+ is required for the functioning of that part of the transport system that converts the chemical energy into pumping energy (Csaky, 1963). In such cases Na^+ will be required internally and a reduction of the external Na^+ concentration would effect the transport system through the concomitant reduction of the internal Na^+ concentration. Crane (1964) however, is of the opinion that Na^+ is required externally for the attachment with the solute transport system and the movement of the solute against its concentration gradient is driven by cotransport of the Na^+ down its electrochemical potential gradient. The metabolic energy in this case will be required for the extrusion of Na^+ from the cell, thereby maintaining a low intracellular Na^+ concentration which creates a favourable gradient for the transport process. In evaluating Crane's model two points have to be considered: first, what is the effect of Na^+ on the affinity of the carrier for the amino acid: second, what is the effect of Na^+ asymmetry on the accumulation of the amino acid.

Crane's model postulates that the attachment of Na^+ to the

transport system will induce an allosteric effect such that this transport system would have a high affinity for the subsequent attachment of the solute. In this case kinetic analysis should show changes in K_m with changes of external Na^+ concentration. Our observations however have demonstrated that the affinity of the carrier for the amino acid did not change as a function of external Na^+ concentration; however the V_{max} was increased when the external Na^+ was increased.

The Na^+ electrochemical gradient as the sole source for driving the amino acid against its concentration gradient when investigated by inhibiting the metabolic energy input by ouabain 10^{-4} M did not corroborate with Crane's model. The amino acid in equilibrated preparations did not move in the direction of the Na^+ gradient. In fact the tissue content of the amino acid did not show any significant change for a period of about 30 min. irrespective of whether there was a Na^+-gradient or not. It might be argued that dissipation of the Na^+-gradient and consequently equilibration with the amino acid would have taken place before the first measurement of the amino acid content have been made (after 5 min.). This however, seems unlikely as the efflux of Na^+ from the cells is delayed in onset (Daniel and Robinson, 1970) and tissue Na^+ was still decreasing during the whole period of observation, an indication that the Na^+ gradient is slowly dissipated. Other evidence against this Na-gradient hypothesis as the driving force comes from our observations on α-aminoisobutyric acid uptake in tissues pretreated with IAA 10^{-3} M. These tissues retained their full ability to concentrate the amino acid against its concentration gra-

dient although the Na^+-pump was inhibited and the Na^+-gradient was markedly reduced. If the Na^+ gradient hypothesis is correct, then inhibition of active Na extrusion should inhibit the active transport process due to disappearance of the Na^+ gradient.

The Na-gradient hypothesis has since received the attention of many investigators as a possible energy input for the transport of many substances across the cell membrane (see Schultz and Curran, 1970 for references). For amino acid transport Vidaver (1964) has shown that the direction of glycine transport in haemolyzed and restored cells was determined exclusively by the direction of the Na^+-gradient, although recent evaluation of the rate equations by numerical integration revealed discrepancies in this assumption which he attributed to an additional energy source or an inappropriate assumption for the rate equations (Vidaver, 1971). Ascites cells depleted of ATP by incubation in 2 mM NaCN accumulated glycine when external Na^+ exceeded that present internally (Eddy, 1968) although such cells were unable to provide oxidative metabolic energy to the transport process. Eddy (1968) has also suggested that the K-gradient might be an additional driving force. The efflux of alanine across the mucosal border of rabbit ileum was dependent on cell Na^+ concentration (Hajjar *et al.*, 1970); alanine efflux was increased when cellular Na^+ was increased. It has been also shown that the efflux of alanine down its concentration gradient was accompanied by net extrusion of Na^+ against its own gradient; an indication of the coupling between Na^+ and alanine efflux out of the cell (Curran *et al.*, 1970).

A detailed investigation performed by Jacquez a..d Schafer (1969) has demonstrated that the Na^+-gradient alone is inadequate to explain the uptake of α-aminoisobutyric acid in ouabain-treated ascites cells under varying Na^+ and K^+ distribution ratios. However the combined effects of the Na^+ and K^+ gradients could in most instances account for the energy required for the accumulation of α-aminoisobubyric acid within the cell.

Experimental evidence against the Na^+-gradient hypothesis has been reported for amino acid uptake in rabbit reticulocytes (Wheeler and Christensen, 1967) and brain slices (Margolis and Lajtha, 1968), and for sugar uptake by isolated intestinal cells (Kimmich, 1970) where the cells were able to concentrate amino acids or sugars from the medium in situations where the internal Na^+ concentration exceeded that present in the external medium. Furthermore the uptake of methionine by intestinal cells was found to be dependent on the intracellular Na^+ concentrations rather than the Na^+-gradient (Newey et al., 1970). Potaschner and Johnstone (1971) have recently shown that in Ehrlich ascites cells the uptake of glycine and methionine was considerably greater in ATP-containing cells than in ATP-depleted cells although Na^+ plus K^+ gradients of similar magnitude were present in both cases. They concluded that the cellular ATP rather the Na^+-gradient is essential for the accumulation of these amino acids.

Our data, although not in favour of the Na-gradient as the sole source of energy for amino acid transport do not exclude the involvement of this element at the present stage.

In considering our findings from the study of structural specificity it has been assumed that the transport system for α-aminoisobutyric acid has specific site(s) for the attachment of the reactive groups and that other amino acids could attach to the same site resulting in reduction or inhibition of α-aminoisobutyric acid uptake. Assuming these considerations the study shows us those amino acids that share the same transport system of α-aminoisobutyric acid as well as the relationship between the various amino acids with respect to that system. Comparisons made between the inhibitory effects of compounds with single differences in structure make it possible to determine those parts of the molecule that are necessary for maximal inhibition.

This study has demonstrated that the transport site for α-aminoisobutyric acid exhibits stereo-specificity; the D-enantiomorphs have no affinity for the site compared to the L-isomers in concentrations used. Moreover it indicates the requirement of the carboxyl group, amino group and side chain for the attachment of the amino acid to the transport site.

Changing the carboxyl group of glycine to the ester or amide form resulted in compounds with no affinity for the transport site. This loss of activity could not apparently be ascribed to steric hindrance by the substituted groups since the COOH group of glycine and $CONH_2$ group of glycinamide are of nearly similar size and the inhibition of α-aminoisobutyric acid uptake was exerted by the former but not by the latter compound. This and other investigations (Paine and

Heinz, 1960; Lin et al., 1962; Spencer et al., 1966; Hajjar and Curran, 1970) indicate the importance of carboxyl group in amino acid transport. A difficulty in these experiments that has to be considered is that the pKa of the amino group of glycine is changed from 9.8 to 7.0 and 7.3 in the ester and amide forms respectively (Perrin, 1965). This means that at physiological pH an appreciable fraction of the ester and amide molecules will have an uncharged α-amino group which might have an effect on the affinity. This point needs further investigation and the role of the charge on the amino group be clarified.

Removal of the amino group as in β-hydroxybutyric acid results in complete abolition of the inhibitory activity on the transport of α - aminoisobutyric acid. Sarcosine, a secondary amino acid inhibited the uptake of α-aminoisobutyric acid to the same degree as did glycine although N-dimethylglycine and betaine were without any significant effect. This confirms the previous findings for amino acid transport in tumour cells (Christensen and Riggs, 1956; Paine and Heinz, 1960) that a primary amino group or certain limited types of secondary amino groups are required for the cellular uptake of the amino acids. This is in contrast to intestinal cells where the transport site has been shown to require a free amino group (Lin et al., 1962; Hajjar and Curran, 1970).

It is apparent that an optimum distance exists between the amino group and carboxyl group for maximum affinity for the transport site. The α-amino acids were found to be inhibitory to the uptake of α - aminoisobutyric acid while the β - and γ- analogues did not exert

any inhibitory response. This indicates the necessary attachment of the amino and carboxyl groups of the amino acid to the α-carbon atom.

The presence of a free α-hydrogen in the L-amino acid is also a requirement for the attachment with the transport site since L-alanine produced more inhibitory effects on the uptake of the labeled α-aminoisobutyric acid than that produced by the non-labeled α-aminoisobutyric acid. Whereas the rate of transport of the α-methylated derivatives have been reported to be increased in tumour cells (Chiriges et al., 1962), the reverse was true for intestinal cells (Lin et al., 1962; Matthews and Laster, 1965).

Previous studies in intestinal preparations have shown that the presence of a net charge in the side chain of the amino acid hinders its active transport (Wiseman, 1953; Lin et al., 1962). Our findings with aspartic acid, glutamic acid and lysine confirm this view that the presence of a positive or negative charge in the side chain abolishes its affinity for the transport site. It was further shown that some amino acids with polar side chains exerted an inhibitory effect on the uptake of α-aminoisobutyric acid similar to that produced by amino acids with non-polar side chains e.g. the inhibition produced by methionine and serine (polar side chain) was comparable with that produced by alanine (non-polar side chain). These findings however differ from previous observations that the side chain must be non-polar in order for the amino acid to have a higher affinity for the transport site (Lin et al., 1962; Hajjar and Curran, 1970). The latter authors have observed that methionine is an exception and has

a much higher affinity than expected but the reason for this was unknown.

It has been shown that the affinity of the amino acid for the transport site was increased as the number of carbon atoms in the non-polar side chain increased (Finch and Hird, 1960; Lin et al., 1962; Hajjar and Curran, 1970; Peterson et al., 1970), suggesting the importance of the lipophilic character of the side chain in the transport process. Our results however have shown that the presence of CH_3 group in the side chain offers maximal affinity for the transport site; affinity decreased with reduction or increase in the length of the side chain.

It might be suggested that a point of attachment for the side chain to the transport site exists at a point adjacent to the β-carbon atom of the amino acid with space limitations lateral to this structure (Oxander and Christensen, 1963b). Branching at the β or γ-carbon atoms of the amino acid or the presence of an aromatic of heterocyclic ring seriously affects the affinity of the amino acid for the site.

The interactions among various amino acids for uptake by Ehrlich ascites cells led Oxander and Christensen (1963a) to postulate the presence of two systems for the transport of neutral amino acids; one designated as "A" or "alanine preferring" system operating for alanine glycine, α-aminoisobutyric acid, serine, threonine and methionine while the other is "L" or "leucine preferring" system for leucine, isoleucine, valine, phenylalanine and also methionine. There is an extensive overlap between these two systems. It seems possible that this

"A" transport system exists in the detrusor muscle since the amino acids which exerted the greatest inhibitory effects on α-aminoisobutyric acid uptake were those which the above authors listed under the "A" or "alanine preferring" system. Further experiments, however are required to characterize the transport systems for neutral amino acids in detrusor muscle.

Since the inhibition by alanine is of the partially competitive type, two possible explanations exist. Either alanine attaches to a site remote from the site of attachment of α-aminoisobutyric acid and by allosteric effect alters the latter's binding ability or alternatively more than one system might exist for the transport of α-aminoisobutyric acid and one of them is competitively inhibited by alanine while the other is not affected. Since transport of amino acids in various tissues has demonstrated the existence of more than one transport site for one amino acid, then the second possibility is more likely.

BIBLIOGRAPHY

Abadom, P.N. and Scholefield, P.G. (1962a). Amino acid transport in brain cortex slices. I. The relation between energy production and the glucose-dependent transport of glycine. Can. J. Biochem. Physiol. 40, 1575-1590.

Abadom, P.N. and Scholefield, P.G. (1962b). Amino acid transport in brain cortex slices. III. The utilization of energy for transport. Can. J. Biochem. Physiol. 40, 1603-1618.

Akedo, H. and Christensen, H.N. (1962a). Transfer of amino acids across the intestine: a new model amino acid. J. Biol. Chem. 237, 113-117.

Akedo, H. and Christensen, H.N. (1962b). Nature of insulin action on amino acid uptake by the isolated diaphragm. J. Biol. Chem. 237, 118-122.

Albers, R.W. (1967). Biochemical aspects of active transport. Ann. Rev. Biochem. 36, 727-756.

Allfrey, V.G., Mendt, R., Hopkins, J.W. and Mirsky, A.E. (1961). Sodium-dependent "transport" reactions in the cell nucleus and their role in protein and nucleic acid synthesis. Proc. Natl. Acad. Sci. U.S. 47, 907-932.

Alvarado, F. (1966). Transport of sugars and amino acids in the intestine: evidence for a common carrier. Science 151, 1010-1013.

Baille, L.A. (1960). Determination of liquid scintillation counting efficiency by pulse height shift. Int. J. Appl. Radiat. Isotopes 8, 1-7.

Baker, R.D. and Copp, D.B. (1965). Effect of dinitrophenol on the pattern of methionine transport along the small intestine of the rat. Experientia 21, 510-511.

Barr, L. and Malvin, R.L. (1965). Estimation of extracellular spaces of smooth muscle using different sized molecules. Am. J. Physiol. 208, 1042-1045.

Begin, N. and Scholefield, P.G. (1963). Amino acid transport by mouse pancreas in vitro. Proc. Can. Federation Biol. Soc. 6, 7.

Begin, N. and Scholefield, P.G. (1964). The uptake of amino acids by mouse pancreas in vitro. I. General characteristics. Biochim. Biophys. Acta 90, 82-89.

Begin, N. and Scholefield, P.G. (1965). The uptake of amino acids by mouse pancreas *in vitro* II. The specificity of the carrier systems. J. Biol. Chem. 240, 332-337.

Berndt, W.O. and Beechwood, E.C. (1965). Influence of inorganic electrolytes and ouabain on uric acid transport. Am. J. Physiol. 208, 642-648.

Beyer, K.H., Wright, L.D., Skeggs, H.R., Russo, H.F. and Shaner, G.A. (1947). Renal clearance of essential amino acids; their competition for reabsorption by the renal tubules. Am. J. Physiol. 151, 202-210.

Bihler, I. and Adamic, S. (1967). The effect of lithium on intestinal sugar transport. Biochim. Biophys. Acta 135, 466-474.

Bihler, I., Hawkins, K.A. and Crane, R.K. (1962). Studies on the mechanism of intestinal absorption of sugars VI. The specificity and other properties of Na^+-dependent entrance of sugars into intestinal tissue under anaerobic conditions, *in vitro*. Biochim. Biophys. Acta, 59, 94-102.

Bingham, J.K., Newey, H. and Smyth, D.H. (1966). Interaction of sugars and amino acids in intestinal transfer. Biochim. Biophys. Acta 130, 281-284.

Bittner, J. and Heinz, E. (1963). Die Wirkung von g-strophantin anf den Glyzintransport in Ehrlich ascites-Tumorzellen. Biochim. Biophys. Acta 74, 392-400.

Blasberg, R. and Lajtha, A. (1965). Substrate specificity of steady state amino acid transport in mouse brain slices. Arch. Biochem. Biophys. 112, 361-377.

Bolton, C. and Wright, G.P. (1937). Absorption of amino acids and their distritutions in body fluids. J. Physiol. 89, 269-286.

Bombara, G. and Bergamini E. (1968). α-aminoisobutyric acid uptake *in vitro* by the rat extensor digitorum longus muscle after denervation and tenotomy. Biochim. Biophys. Acta 150, 226-236.

Born, G.V.R. (1962). The fate of 5 hydroxytryptamine in a smooth muscle and in connective tissue. J. Physiol 161, 160-174.

Boulpaep, E. (1963). Permeability of heart muscle to choline. Arch. Int. Physiol. 71, 623-625.

Bozler, E. (1966). Movement of non-electrolytes in intact and extracted muscle fibres. Biochem. Z. 345, 101-107.

Bozler, E. and Lavine, D. (1958). Permeability of smooth muscle. Am. J. Physiol. 195, 45-49.

Brading, A.F. and Jones, A.W. (1969). Distribution and kinetics of CoEDTA in smooth muscle and its use as an extracellular marker. J. Physiol. 200, 387-401.

Bray, G.A. (1960). A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Analyt. Biochem. 1, 279-285.

Bricker, N.S. and Klahr, S. (1966). Effect of dinitrophenol and oligomycin on the coupling between anoerobic metabolism and anoerobic sodium transport by the isolated turtle bladder. J. Gen. Physiol. 49, 483-499.

Brown, D.M. and Michael, A.F. (1971). Effects of Ca^{2+} and Mg^{2+} upon amino acid transport in rat renal cortex slices. Biochim. Biophys. Acta 233, 215-221.

Brown, D.A., Stumpf, W.E. and Roth, L.J. (1969). Location of radioactively labelled extracellular fluid indicators in nervous tissue by autoradiography. J. Cell. Sci. 4, 265-288.

Burnstock, G. (1970). Structure of smooth muscle and its innervation. In Smooth Muscle, ed. Bulbring, E., Brading, A.F., Jones, A.W. and Tomita, T. Edward Arnold (Publishers) Ltd., London, p. 1-69.

Burnstock, G., Holman, M.E. and Prosser, C.L. (1963). Electrophysiology of smooth muscle. Physiol. Rev. 43, 482-527.

Bush, E.T. (1963). General applicability of the channels ratio method of measuring liquid scintillation counting efficiencies. Analyt. Chem. 35, 1024-1029.

Caesar, R., Edward, G.A. and Ruska, H. (1957). Architecture and nerve supply of mammalian smooth muscle tissue. J. Biophys. Biochem. Cytol. 3, 867-878.

Cammarata, P.S. and Cohen, P.P. (1950). The scope of the transamination reaction in animal tissues. J. Biol. Chem. 187, 439-452.

Casteels, R. (1966). The action of ouabain on the smooth muscle cells of the guinea pig's taenia coli. J. Physiol. 184, 131-142.

Casteels, R. (1970). The relation between the membrane potential and the ion distribution in smooth muscle cells. In Smooth Muscle, ed. Bulbring, E., Brading, A.F., Jones, A.W. and Tomita, T. Edward Arnold (Publishers) Ltd., London, p. 70-99.

Caston, J.D. and Singer, M. (1969). Amino acid uptake and incorporation into macromolecules of peripheral nerves. J. Neurochem. 16, 1309-1318.

Charnock, J.S. and Opit, L.J. (1968). Membrane metabolism and ion transport. In The Biological Basis of Medicine, ed. Bittar, E.E., Vol. I, Academic press.

Charnock, J.S. and Post, R.L. (1963). Studies of the mechanism of cation transport. I. The preparation and properties of a cation stimulated adenosine-triposphatase from guinea pig kidney cortex. Aust. J. Exp. Biol. Med. Sci. 41, 675-686.

Chase, B.W. and Lewis, H.B. (1934). Comparative studies of the metabolism of amino acids VI. The rate of absorption of leucine Valine and their isomers from the gastrointestinal tract of the white rat. J. Biol. Chem. 106, 315-321.

Chez, R.A., Palmer, R.R., Schultz, S.G. and Curran, P.F. (1967). Effect of inhibitors on alanine transport in isolated rabbit ileum. J. Gen. Physiol. 50, 2357-2375.

Chez, R.A., Schultz, S.G. and Curran, P.F. (1966). Effect of sugars on transport of alanine in intestine. Science 153, 1012-1013.

Chirigos, M.A., Fanning, G.R. and Guroff, G. (1962). Effects of amino acids, analogs, and certain other agents in relation to tyrosine transport in Sarcoma 37 ascites cells. Cancer Res. 22, 1349-1355.

Christensen, H.L. (1959). Active transport with special reference to the amino acids. Perspectives in Biology and Medicine 2, 228-242.

Christensen, H.N., Akedo, H., Oxander, D.L. and Winter, C.G. (1962). On the mechanism of amino acid transport into cells. In Amino Acid Pools, ed. Holden, J.T. New York, Elsevier Publishing Company, p. 527-538.

Christensen, H.N., Cushing M.K. and Streicher, J.A. (1949). Concentration of amino acids by the excised diaphragm suspended in artificial media II. Inhibition of the concentration of glycine by amino acids and related substances. Arch. Biochem. 23, 106-110.

Christensen, H.N., Handlogten, M.E. and Thomas, E.L. (1969). Na^+-facilitated reactions of neutral amino acids with a cationic amino acid transport system. Proc. Nat. Acad. Sci. U.S.A. 63, 948-955.

Christensen, H.N. and Liang, M. (1966). On the nature of the "non-saturable" migration of amino acids into Ehrlich ascites cells and into rat jejunum. Biochim. Biophys. Acta, 112, 524-531.

Christensen, H.N., Liang, M. and Archer, E.G. (1967). A distinct Na^+ requiring transport system for alanine, serine, cysteine and similar amino acids. J. Biol. Chem. 242, 5237-5246.

Christensen, H.N. and Riggs, T.R. (1952). Concentrative uptake of amino acids by the Ehrlich mouse ascites carcinoma cell. J. Biol. Chem. 194, 57-68.

Christensen, H.N. and Riggs, T.R. (1956). Structural evidence for chelation and Schiff's base formation on amino acid transfer into cells. J. Biol. Chem. 220, 265-278.

Christensen, H.N., Riggs, T.R., Fischer, H. and Palatine, I.M. (1952a). Intense concentration of α, γ-diamino-butyric acid by cells. J. Biol. Chem. 198, 17-22.

Christensen, H.N., Riggs, T.R., Fischer, H. and Palatine, I.M. (1952b). Amino acid concentration by a free cell neoplasm: relation among amino acids. J. Biol. Chem. 198, 1-15.

Christensen, H.N., Riggs, T.R. and Ray, N.E. (1952c). Concentrative uptake of amino acids by erythrocytes *in vitro*. J. Biol. Chem. 194, 41-51.

Christensen, H.N. and Streicher, J.A. (1949). Concentration of amino acids by the excised diaphragm suspended in artificial media I. Maintenance and inhibition of the concentrating activity. Arch. Biochem. 23, 96-105.

Clarke, E.W., Gibson, Q.H., Smyth, D.H. and Wiseman, G. (1951). Selective absorption of amino acids from thiry vella loops. J. Physiol. 112, p. 46.

Cohen, S.R., Blasberg, R., Levi, G. and Lajtha, A. (1968). Compartmentation of the inulin space in mouse brain slices. J. Neurochem. 15, 707-720.

Cohen, L.L. and Huang, K.C. (1964). Intestinal transport of tryptophan and its derivatives. Am. J. Physiol. 206, 647-652.

Cohen, S.R., Stampleman, P.F. and Lajtha, A. (1970). The temperature-dependent compartmentation of the extracellular space in mouse brain slices as revealed by the markers: Inulin, Sucrose, D-Mannitol, D-Sorbitol and Sulfate. Brain Res. 21, 419-434.

Conway, E.J. (1957). Nature and significance of concentration relations of potassium and sodium ions in skeletal muscle. Physiol. Rev. 37, 84-132.

Cook, D.A. and Taylor, G.S. (1971). The use of the APL/360 system in Pharmacology. A computer assisted analysis of efflux data. Comp. Biomed. Res. 4, 7168-7177.

Crane, R.K. (1964). Uphill outflow of sugar from intestinal epithelial cells induced by reversal of the Na^+ gradient: its significance for the mechanism of Na^+-dependent active transport. Biochem. Biophys. Res. Commun. 17, 481-485.

Crane, R.K., Forstner, G. and Eichholz, A. (1965). Studies on the mechanism of the intestinal absorption of sugars. X. An effect of Na^+ concentration on the apparent Michaelis constants for intestinal sugar transport, in vitro. Biochim. Biophys. Acta, 109, 467-477.

Crawhall, J.C. and Segal, S. (1968). Transport of some amino acids and sugars in rat liver slices. Biochim. Biophys. Acta 163, 163-170.

Csaky, T.Z. (1963). A possible link between active transport of electrolytes and non-electrolytes. Federation Proc. 22, 3-7.

Csaky, T.Z. and Hara, Y. (1965). Inhibition of active intestinal sugar transport by digitalis. Am. J. Physiol. 209, 467-427.

Curran, P.F., Hajjar, J.J. and Glynn, I.M. (1970). The Na-alanine interaction in rabbit ileum: effect of alanine on Na fluxes. J. Gen. Physiol. 55, 297-308.

Curran, P.F., Schultz, S.G., Chez, R.A. and Fuisz, R.E. (1967). Kinetic relations of the Na-amino acid interaction at the mucosal border of the intestine. J. Gen. Physiol. 50, 1261-1286.

Daniel, E.E. (1958). Smooth muscle electrolytes. Can. J. Biochem. Physiol. 36, 805-818.

Daniel, E.E. (1963). Potassium movements in rat uterus studied in vitro I. Effects of temperature. Can. J. Biochem. Physiol. 41, 2065-2084.

Daniel, E.E. (1965). Effects of intra-arterial perfusions on electrical activity and electrolyte contents of dog small intestine. Canad. J. Physiol. Pharmacol. 43, 551-577.

Daniel, E.E. and Robinson, K. (1970). Sodium exchange and net movement in rat uteri at 25°C. Can. J. Physiol. Pharmacol. 48, 598-624.

Daniels, V.G., Dawson, A.G., Newey, H. and Smyth, D.H. (1969a). Effect of carbon chain length and amino group position on neutral amino acid transport systems in rat small intestine. Biochim. Biophys. Acta 173, 575-577.

Daniels, V.G., Newey, H. and Smyth, D.H. (1969b). Stereochemical specificity of neutral amino acid transfer systems in rat small intestine. Biochim. Biophys. Acta 183, 637-639.

Danielson Bo, G. (1964). The distribution of some electrolytes in the heart. Acta Physiol. Scand. 62 supp.

Dawson, H. and Danielli, J. (1943). The permeability of natural membranes. Cambridge University press, p. 53-57.

Day, T.D. (1950). Connective tissue permeability and the mode of action of hyaluronidase Nature, Lond. 166, 785-786.

Day, T.D. (1952). The permeability of interstitial connective tissue and the nature of interfibrillary substance. J. Physiol. 117, 1-8.

DeAlmedia, D.F., Chain, E.B. and Pocchiari, F. (1965). Effect of ammonium and other ions on the glucose dependent active transport of L-Histidine in slices of rat brain cortex. Biochem. J. 95, 793-796.

Dick, D.A.T. and Lea, E.J.A. (1964). Na fluxes in single toad oocytes with special reference to the effect of external and internal Na concentration on Na efflux. J. Physiol. 174, 55-90.

Dixon, M. (1953). The determination of enzyme inhibitor constants. Biochem. J. 55, 170-171.

Dryden, E.E. and Manery J.F. (1970). Preparation of tissue and fluid samples for determination of tissue spaces using sorbitol and/or inulin labelled with carbon-14 or Tritium. Anal. Biochem. 35, 384-392.

Durbin, R.P. and Monson, R.R. (1961). Ionic composition and permeability of smooth muscle. Federation Proc. 20, 134.

Eavenson, E. and Christensen, H.N. (1967). Transport systems for neutral amino acids in the pigeon erythrocyte. J. Biol. Chem. 242, 5386-5396.

Eddy, A.A. (1968). The effects of varying the cellular and extracellular concentrations of sodium and potassium ions on the uptake of glycine by mouse ascites tumour cells in the presence and absence of sodium cyanide. Biochem. J. 108, 489-498.

Eddy, A.A. and Hogg, M.C. (1969). Further observations on the inhibitory effect of extracellular potassium ions on glycine uptake by mouse ascites tumour cells. Biochem. J. 114, 807-814.

Eddy, A.A., Hogg, C. and Reid, M. (1969). Ion gradients and the accumulation of various amino acids by mouse ascites tumour cells depleted of adenosine triphosphate. Biochem. J. 112, 11P-12P.

Eddy, A.A., Mulcahy, M.F. and Thomson, P.J. (1967). The effects of sodium ions and potassium ions on glycine uptake by mouse ascites tumour cells in the presence and absence of selected metabolic inhibitors. Biochem. J. 103, 863-876.

Eisenman, G. (1961). On the elementary atomic origin of equilibrium ionic specificity. In Symposium on Membrane Transport and Metabolism, ed. Kleinzeller, A. and Kotyk, A., New York, Academic press, p. 163-179.

Ellis, D.B. and Scholefield, P.G. (1961). The effects of uncoupling agents on the uptake and incorporation of glycine by transplantable tumours. Cancer Res. 21, 650-657.

Epstein, F.H. and Whittam, R. (1966). The mode of inhibition by calcium of cell membrane adenosine triphosphatase activity. Biochem. J. 99, 232-238.

Evered, D.F. and Randall, H.G. (1962). The absorption of amino acid derivatives of nitrogen mustard from rat intestine in vitro. Biochem. Pharmacol. 11, 371-376.

Fenn, W.O. (1936). Electrolytes in muscle. Physiol. Rev. 16, 405-487.

Field, M. Schultz, S.G. and Curran, P.F. (1967). Alanine transport across isolated rabbit ileum. Biochim. Biophys. Acta 135, 236-243.

Finerman, G.M.A. and Rosenberg, L.E. (1966). Amino acid transport in bone. Evidence for separate transport system for neutral amino and imino acid. J. Biol. Chem. 241, 1487-1493.

Fox, M., Thier, S., Rosenberg, L. and Segal, S. (1964). Ionic requirements for amino acid transport in the rat kidney cortex slice I. Influence of extracellular ions. Biochim. Biophys. Acta 79, 167-176.

Frankenhaeuser, B. and Hodgkin, A.L. (1957). The action of calcium on the electrical properties of squid axons. J. Physiol. 137, 218-244.

Frizzell, R.A. and Schultz, S.G. (1970). Effects of monovalent cations on the sodium-alanine interaction in rabbit ileum. Implication of anionic groups in sodium binding. J. Gen. Physiol. 56, 462-490.

Glynn, I.M. (1964). The action of cardiac glycosides on ion movements. Pharmacol. Rev. 16, 381-407.

Goldner, A.M., Schultz, S.G. and Curran, P.F. (1969). Sodium and sugar fluxes across the mucosal border of rabbit ileum. J. Gen. Physiol. 53, 362-383.

Gonda, O. and Quastel, J.H. (1962). Effects of ouabain on cerebral metabolism and transport mechanisms in vitro. Biochem. J. 84, 394-406.

Goodford, P.J. (1967). The radioactive cobalticyanide ion $^{60}Co(CN)_6^{3-}$ as an extracellular marker of smooth muscle. J. Physiol. 191, 111P.

Goodford, P.J. (1970). Ionic interactions in smooth muscle. In Smooth Muscle, ed. Bulbring, E., Brading, A.F., Jones, A.W. and Tomita, T. Edward Arnold (Publishers) Ltd., London, p. 100-121.

Goodford, P.J. and Hermansen, K. (1961). Sodium and potassium movements in the unstriated muscle of the guinea pig taenia coli. J. Physiol. 158, 426-448.

Goodford, P.J. and Leach, E.H. (1964). The extracellular space of intestinal smooth muscle. J. Physiol. 175, 38P.

Goodford, P.J. and Leach E.H. (1966). Extracellular space of the smooth muscle of the guinea pig taenia coli. J. Physiol. 186, 1-10.

Goodford, P.J. and Lullmann, H. (1962). The uptake of ethanesulphonate-^{35}S ion by muscular tissue. J. Physiol. 161, 54-61.

Goodman, H.M. (1966). Alpha aminoisobutyric acid transport in adipose tissue. Am. J. Physiol. 211, 815-820.

Griffin, D.M. and Szego, C.M. (1968). Adenosine 3', 5'-monophosphate stimulation of uterine amino acid uptake in vitro. Life Sciences, Vol. 7, Part II, 1017-1023.

Gurroff,G., King, W. and Udenfriend, S. (1961). The uptake of tyrosine by rat brain in vitro. J. Biol. Chem. 236, 1773-1777.

Gurroff,G. and Udenfriend, S. (1962). Uptake of tyrosine by brain in vitro. In amino acid Pools, ed. Holden, J.T., New York, Elsevier Publishing Co., p. 545-553.

Hajjar, J.J. and Curran, P.F. (1970). Characteristics of the amino acid transport system in the mucosal border of rabbit ileum. J. Gen. Physiol. 56, 673-691.

Hajjar, J.J., Lamont, A.S. and Curran, P.F. (1970). The Na-alanine interaction in rabbit ileum: effect of Na on alanine fluxes. J. Gen. Physiol. 55, 277-296.

Hauser, G. (1969). Myo-inositol transport in slices of rat kidney cortex II. Effect of ionic composition of the medium. Biochim. Biophys. Acta 173, 267-276.

Haust, M.D. (1965). Fine fibrils of extracellular space (Microfibrils). Am. J. Path. 47, 1113-1137.

Heinz, E. (1954). Kinetic studies on the influx of glycine-1-^{14}C into the Ehrlich mouse ascites carcinoma cell. J. Biol. Chem. 211, 781-790.

Heinz, E. (1962). Some remarks on active transport and exchange diffusion of amino acids in Ehrlich cells. In Amino Acid Pools, ed. Holden, J.T., New York, Elsevier Publishing Company, p. 539-544.

Heinz, E. and Walsh, P.M. (1958). Exchange diffusion transport and intracellular level of amino acids in Ehrlich carcinoma cells. J. Biol. Chem. 233, 1488-1493.

Helmreich, E. and Kipnis, D.M. (1962). Amino acid transport in lymph node cells. J. Biol. Chem. 237, 2582-2589.

Hempling, H.G. and Hare, D. (1961). The effect of glycine transport on potassium fluxes in the Ehrlich mouse ascites tumour cell. J. Biol. Chem. 236, 2498-2502.

Herra, M.G. and Renold, A.E. (1960). Hormonal effects on glycine metabolism in rat epididymal adipose tissue. Biochim. Biophys. Acta 44, 165-167.

Hindmarsh, J.T., Kilby, D. and Wiseman, G. (1966). Effect of amino acids on sugar absorption. J. Physiol. 186, 166-174.

Hober, R. and Hober J. (1937). Experiments on the absorption of organic solutes in the small intestine of rats. J. Cell and Comp. Physiol. 10, 401-422.

Huxley, A.F. (1960). Compartmental methods of kinetic analysis. Comar, C.L. and Bronner, F. in Mineral Metabolism, Vol. I, Part A, New York, Academic Press, p. 163-166.

Inui, Y. and Christensen, H.N. (1966). Discrimination of single transport systems. The Na^+-sensitive transport of neutral amino acids in the Ehrlich cells. J. Gen. Physiol. 50, 203-224.

Jacquez, J.A. and Schafer, J.A. (1969). Na^+ and K^+ electrochemical potential gradients and the transport of α-aminoisobutyric acid in Ehrlich ascites tumour cells. Biochim. Biophys. Acta 193, 368-383.

Jacquez, J.A., Sherman, J.H. and Terris, J. (1970). Temperature dependence of amino acid transport in Ehrlich ascites cells: with results which bear on the A-L distinction. Biochim. Biophys. Acta 203, 150-166.

Johnstone, R.M. (1959). Arginine uptake and arginase activity in Ehrlich ascites carcinoma cells. Can. J. Biochem. Physiol. 37, 589-598.

Johnstone, R.M. and Scholefield, P.G. (1959). The influence of amino acids and antimetabolites on glycine retention by Ehrlich ascites carcinoma cells. Cancer Res. 19, 1140-1149.

Johnstone, R.M. and Scholefield, P.G. (1965). Amino acid transport in tumour cells. Advan. Cancer Res. 9, 143-226.

Jones, A.W. (1970). Factors affecting sodium exchange and distribution in rabbit myometrium. Physiol. Chem. Physics 2, 79-95.

Jones, A.W. and Karreman, G. (1969). Ion exchange properties of canine carotid artery. Biophys. J. 9, 884-909.

Keynes, R.D. and Swan, R.C. (1959). The effect of external sodium concentration on the sodium fluxes in frog skeletal muscle. J. Physiol. 147, 591-625.

Kimmich, G.A. (1970). Active sugar accumulation by isolated intestinal epithelial cells. A new model for sodium-dependent metabolite transport. Biochem. 9, 3669-3677.

Kipnis, D.M. and Parrish, J.E. (1965). Role of Na^+ and K^+ on sugar (2-deoxyglucose) and amino acid (α-aminoisobutyric acid) transport in striated muscle. Federation Proc. 24, 1051-1059.

Kipnis, D.M., Reiss, E. and Helmreich, E. (1961). Functional heterogeneity of the intracellular amino acid pool in mammalian cells. Biochim. Biophys. Acta 51, 519-524.

Kleinzeller, A., Knotkova, A. and Nedvidkova, J. (1968). The effect of calcium ions in the steady state ionic distribution in kidney cortex cells. J. Gen. Physiol. 51, 3265-3345.

Kostyo, J.L. and Schmidt, J.E. (1963). Inhibitory effects of cardiac glycosides and adrenal steroids on amino acid transport. Am. J. Physiol. 204, 1031-1038.

Kromphardt, H., Grobecker, H., Ring, K. and Heinz, E. (1963). Uber den einfluss von alkali-ionen auf den glycintransport in Ehrlich ascites tumorzellen. Biochim. Biophys. Acta 74, 549-551.

Lahiri, S. and Lajtha, A. (1964). Cerebral amino acid transport *in vitro* I. Some requirements and properties of uptake. J. Neurochem. 11, 77-86.

Lane, B.P. and Rhodin, J.A.G. (1964). Cellular inter-relationships and electrical activity in two types of smooth muscle. J. Ultrastruct. Res. 10, 470-488.

Lang, K. and Oster, H. (1953). Untersuchugen uber den stoffwechsel der α-aminobuttersaure and der α-aminoisobuttersaure. Biochem. Z. 324, 443-446.

Lardy, H.A. and Wellman, H. (1953). The catalytic effect of 2,4-dinitrophenol on adenosinetriphosphate hydrolysis by cell particles and soluble enzymes. J. Biol. Chem. 201, 357-370.

Laws, J.O. and Stickland, L.H. (1967). The time-course of the synthesis of adenosinetriphosphate in Ehrlich ascites-tumour cells, and the effects of 2,4-dinitrophenol. Biochem. J. 104, 158-164.

Lesch, M., Gorlin, R. and Sonnenblick, E.H. (1970). Myocardial amino acid transport in the isolated rabbit right ventricular papillary muscle. General characteristics and effects of passive stretch. Circulation Res., Vol. XXVII, 445-460.

Levi, G. (1969). Different estimates of tissue extracellular space using [Carboxyl-^{14}C]-Inulin from different sources. Analyt. Biochem. 32, 348-353.

Lin, E.C.C., Hagihira, H. and Wilson, T.H. (1962). Specificity of the transport system for neutral amino acids in hamster intestine. Am. J. Physiol. 202, 919-925.

Luck, J.M. (1928). The metabolism of amino acids. J. Biol. Chem. 77, 13-26.

Manery, J.F. (1966). Effects of Ca ions on membranes. Fed. Proc. 25, 1804-1810.

Margolis, R.K. and Lajtha, A. (1968). Ion dependence of amino acid uptake in brain slices. Biochim. Biophys. Acta 163, 374-385.

Matthews, D.M. and Laster, L. (1965). Kinetics of intestinal active transport of five neutral amino acids. Am. J. Physiol. 208, 593-600.

Meyer, K., Davidson, E., Linker, A. and Hoffman, P. (1956). The acid mucopolysaccharides of connective tissue. Biochim. Biophys. Acta 21, 506-518.

Munck, B.G. (1967). Intestinal transport of sugars and amino acids in rats' small intestine *in vitro*. Lack of evidence of competitive inhibition between the two groups. Federation Proc. 26, 541.

Munck, B.G. (1968a). Amino acid transport by the small intestine of the rat. Effects of glucose on transintestinal transport of proline and valine. Biochim. Biophys. Acta 150, 82-91.

Munck, B.G. (1968b). Amino acid transport by the small intestine of the rat. Evidence against interactions between sugars and amino acids at the carrier level. Biochim. Biophys. Acta 156, 192-194.

Munck, B.G. and Schultz, S.G. (1969). Lysine transport across isolated Rabbit ileum. J. Gen. Physiol 53, 157-182.

Munson, J. and Paton, D.M. (1971). Metabolic requirements for Na^+ pumping in rabbit detrusor muscle. Proc. Can. Federation Biol. Soc. 14, 172.

Meyers, D.K. and Slater, E.C. (1957). The enzymatic hydrolysis of adenosine triphosphatase by liver mitochondria I. Activities at different P^H values. Biochem, J. 67,558-572.

Newey, H., Rampone, A.J. and Smyth, D.H. (1970). The relation between L-methionine uptake and sodium in rat small intestine *in vitro*. J. Physiol. 211, 539-549.

Newey, H. and Smyth, D.H. (1964a). The transfer system for neutral amino acids in the rat small intestine. J. Physiol. 170, 328-343.

Newey, H. and Smyth, D.H. (1964b). Effects of sugars on intestinal transfer of amino acids. Nature 202, 400-401.

Nelson, K.M. and Lerner, J. (1970). A distinct Na^+-dependent glycine transport system in avian small intestine. Biochim. Biophys. Acta 203, 434-444.

Neame, K.D. and Smith, S.E. (1965). Uptake of D- and L-alanine by rat brain slices. J. Neurochem. 12, 87-91.

Newey, H., Sanford, P.A. and Smyth, D.H. (1968). Some effects of ouabain and potassium on transport and metabolism in rat small intestine. J. Physiol. (London) 194, 237-248.

Nicholls, J.G. and Wolfe, D.E. (1967). Distribution of ^{14}C-labelled sucrose, inulin and dextran in extracellular spaces and in cells of the leech central nervous system. J. Neurophysiol. 30, 1574-1592.

Noall, M.W. and Allen, W.M. (1961). Early stimulation of amino acid penetration in rabbit uterus. J. Biol. Chem. 236, 2987-2990.

Noall, M.W., Riggs, T.R., Walker, L.M. and Christensen, H.N. (1957). Endocrine control of amino acid transfer. Science 126, 1002-1005.

Noujaim, A., Eddis, C. and Wiebe, L. (1971). Precision of some quench correction methods in liquid scintillation counting. In Org. Scintillators and Liquid Scintillation Counting, New York, Academic Press, p. 705-717.

Ogston, A.G. and Phelps, C.F. (1961). The partition of solutes between buffer solutions and soultions containing hyaluronic acid. Biochem. J. 78, 827-833.

Oxander, D.L. and Christensen, H.N. (1963a). Evidence for two types of mediation of neutral amino acid transport in Ehrlich cells. Nature 197, 765-767.

Oxander, D.L. and Christensen, H.N. (1963b). Distinct mediating systems for the transport of neutral amino acids by the Ehrlich cells. J. Biol. Chem. 238, 3686-3699.

Page, E. (1962). Cat heart muscle in vitro III. The extracellular space. J. Gen. Physiol. 46, 201-213.

Paine, C.M. and Heinz, E. (1960). The structural specificity of the glycine transport system of Ehrlich carcinoma cells. J. Biol. Chem. 235, 1080-1085.

Palaty, V., Gustafson, B. and Friedman, S.M. (1969). Sodium binding in arterial wall. Can. J. Physiol. Pharmacol. 47, 763-770.

Parrish, J.E. and Kipnis, D.M. (1964). Effect of Na^+ on sugar and amino acid transport in striated muscle. J. Clin. Invest. 43, 1994-2002.

Paterson, C.A. (1968). Lens extracellular space: discrepancies using [^{14}C]-inulin obtained from different commercial sources. Exptl. Eye Res. 7, 431-433.

Paton, D.M. (1968). Effect of metabolic inhibitors on contraction of rabbit detrusor muscle. Brit. J. Pharmacol. 34, 493-498.

Paton, D.M. (1969). Control of Na^+ and K^+ content of rabbit detrusor muscle. Clin. Res. 17, 130.

Perl, W. (1960). A method for curve-fitting by exponential function. Int. J. Appl. Radiat. Isotop. 8, 223-227.

Perrin, D.D. (1965). Dissociation Constants of Organic Bases in Aqueous Solution. Butterworth and Co. (Publishers) Ltd., London.

Persoff, D.A. (1960). A comparison of methods for measuring efflux of labelled potassium from contracting rabbit atria. J. Physiol. 152, 354-366.

Peterson, S.C., Goldner, A.M. and Curran, P.F. (1970). Glycine transport in rabbit ileum. Am. J. Physiol. 219, 1027-1032.

Potashner, S. and Johnstone, R.M. (1970). Cations, transport and exchange diffusion of methionine in Ehrlich ascites cells. Biochim. Biophys. Acta 203, 445-456.

Potashner, S.J. and Johnstone, R.M. (1971). Cation gradients, ATP and amino acid accumulation in Ehrlich ascites cells. Biochim. Biophys. Acta 233, 91-103.

Prosser, C.L., Burnstock, G. and Kahn, J. (1960). Conduction in smooth muscle: comparative structural properties. Am. J. Physiol. 199, 545-552.

Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960). Partial resolution of the enzymes catalyzing oxidative phosphorylation I. Purification and properties of soluble, dinitrophenol-stimulated adenosine triphosphatase. J. Biol. Chem. 235, 3322-3329.

Reddy, D.V.N. and Kinsey. V.E. (1962). Transport of alpha aminoisobutyric acid into ocular fluids and lens. Investigative Ophthalmology 1, 41-51.

Reiser, S. and Christiansen, P.A. (1967). Intestinal transport of valine as affected by ionic environment. Am. J. Physiol. 212, 1297-1302.

Renkin, E.M. (1961). Permeability of frog skeletal muscle cells to choline. J. Gen. Physiol. 44, 1159-1164.

Rifkin, R.J. (1965). In vitro inhibition of Na^+, K^+ and Mg^{2+} ATPases by Mono, Di and Trivalent cations. Proc. Soc. Exptl. Biol. Med. 120, 802-804.

Riggs, T.R., Christensen, H.N. and Palatine, I.M. (1952). Concentrating activity of reticulocytes for glycine. J. Biol. Chem. 194, 53-55.

Riggs, T.R., Coyne, B.A. and Christensen, H.N. (1954). Amino acid concentration by a free cell neoplasm. Structural influences. J. Biol. Chem. 209, 395-411.

Riggs, T.R., Coyne, B.A. and Christensen, H.N. (1958). Potassium migration and amino acid transport. J. Biol. Chem. 233, 1479-1484.

Riggs, T.R., Pan, M.W. and Peng, H.W. (1968). Transport of amino acids into the estrogen-primed uterus I. General characteristics of the uptake in vitro. Biochim. Biophys. Acta. 150, 92-103.

Robertson, J.S. (1957). Theory and use of tracers in determining transfer rates in biological systems. Physiol. Rev. 37, 133-154.

Rosenberg, L.E., Blair, A. and Segal, S. (1961). Transport of amino acids by slices of rat kidney cortex. Biochim. Biophys. Acta 54, 479-488.

Rosenberg, I.H., Coleman, A.L. and Rosenberg, L.E. (1965). The role of sodium ion in the transport of amino acids by the intestine. Biochim. Biophys. Acta 102, 161-171.

Rosenbusch, J.P., Flanagan, B. and Nichols, G. Jr. (1967). Active transport of amino acids into bone cells. Biochim. Biophys. Acta 135, 732-740.

Roskoski, R. Jr., and Steiner, D.F. (1967a). Cyclohexemide and actinomycin D inhibition of estrogen-stimulated sugar and amino acid transport in rat uterus. Biochim. Biophys. Acta 135, 347-349.

Roskoski, R. Jr. and Steiner, D.F. (1967b). The effect of oestrogen on amino acid transport in rat uterus. Biochim. Biophys. Acta 135, 727-731.

Rubinstein, L. and Ahren, K. (1969). Uptake of α-aminoisobutyric acid (AIB) in isolated ovaries from androgenized rats. Endocrinology 84, 803-807.

Sandow, A.A. (1939). A geometrical determination of the extracellular space in muscle. Soc. Exp. Biol. Med. 42, 772-778.

Scharff, R. and Wool, I.G. (1965). Accumulation of amino acids in muscle of perfused rat heart. Effect of insulin in the presence of puromycin. Biochem. J. 97, 272-276.

Schultz, S.G. and Curran, P.F. (1970). Coupled transport of sodium and organic solutes. Physiol. Rev. 50, 637-718.

Schultz, S.G., Curran, P.F., Chez, R.A. and Fuisz, R.E. (1967). Alanine and sodium fluxes across mucosal border of rabbit ileum. J. Gen. Physiol. 50, 1241-1260.

Schultz, S.G., Fuisz, R.E. and Curran, P.F. (1966). Amino acid and sugar transport in rabbit ileum. J. Gen. Physiol. 49, 849-866.

Schultz, S.G. and Zalusky, R. (1964a). Ion transport in isolated rabbit ileum I. Short-circuit current and Na fluxes. J. Gen. Physiol. 47, 567-584.

Schultz, S.G. and Zalusky, R. (1964b). Ion transport in isolated rabbit ileum II. The interaction between active sodium and active sugar transport. J. Gen. Physiol. 47, 1043-1059.

Scriver, C.R. and Mohyuddin, F. (1968). Amino acid transport in kidney. Heterogeneity of α-aminoisobutyric uptake. J. Biol. Chem. 243, 3207-3213.

Segal, S. and Crawhall, J.C. (1968). Characteristics of cystine and cysteine transport in rat kidney cortex slices. Proc. Nat. Acad. Sci. U.S.A. 59, 231-237.

Sharma, S.K., Johnstone, R.M. and Quaster, J.H. (1964). Corticosteroids and ascorbic acid transport in adrenal cortex in vitro. Biochem. J. 92, 564-573.

Simkin, J.L. (1959). Protein Biosynthesis. Ann. Rev. Biochem. 28, 145-170.

Skou, J.C. (1960). Further investigations of a magnesium requiring sodium activated adenosine triphosphatase. Biochim. Biophys. Acta 24, 6-23.

Skou, J.C. (1962). Preparation from mammalian brain and kidney of the enzyme system involved in active transport of sodium and potassium. Biochim. Biophys. Acta 58, 314-325.

Skou, J.C. (1965). Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. Physiol. Rev. 45, 596-617.

Slater, E.C. (1963). Uncouplers and inhibitors of oxidative phosphorylation. In Metabolic Inhibitors, ed. Hochster, R.M. and Quastel, J.H. Academic Press, p. 503-516.

Smith, I. and Segal, S. (1968). The influence of size of rat kidney cortex slices on the accumulation of amino acids. Biochim. Biophys. Acta 163, 281-283.

Spencer, R.P., Brody, K.R. and Vishno, F.E. (1966). Role of the carboxyl group in intestinal amino acid transport. Biochim. Biophys. Acta 117, 410-415.

Sterling, W.R. and Henderson, J.F. (1963). Studies on the mechanism of action of 1-amino cyclopentane-1-carboxylic acid. Biochem. Pharmacol. 12, 303-316.

Stern, J.R., Eggleston, L.V., Hems, R. and Krebs, H.A. (1949). Accumulation of glutamic acid in isolated brain tissue. Biochem. J. 44, 410-418.

Stirling, C.E. and Kinter, W.B. (1967). High resolution autoradiography of galactase H^3 accumulation in rings of hamster intestine. J. Cell. Biol. 35, 585-604.

Takagaki, G., Hirano, S. and Nagata, Y. (1959). Some observations on the effects of D-glutamate on the glucose metabolism and the accumulation of potassium ions in brain cortex slices. J. Neurochem. 4, 124-134.

Tenenhouse, A. and Quastel, J.H. (1960). Amino acid accumulation in Ehrlich ascites carcinoma cells. Can. J. Biochem. Physiol. 38, 1311-1325.

Tews, J.K. and Harper, A.E. (1969). Transport of nonmetabolizable amino acids in rat liver slices. Biochim. Biophys. Acta 183, 601-610.

Thier, S.O. (1968). Amino acid accumulation in the toad bladder: relationship to transepithelial sodium transport. Biochim. Biophys. Acta 150, 253-262.

Thier, S., Fox, M., Rosenberg, L. and Segal, S. (1964). Hexose inhibition of amino acid uptake in the rat kidney cortex slice. Biochim. Biophys. Acta 93, 106-115.

Touabi, M. and Jeanrenaud, B. (1969). α-aminoisobutyric acid uptake in isolated mouse fat cells. Biochim. Biophys. Acta 173, 128-140.

Tsukada, Y., Nagata, Y., Hirano, S. and Matsutani, T. (1963). Active transport of amino acid into cerebral cortical slices. J. Neurochem. 10, 241-256.

Van Slyke, D.D. and Meyer, G.M. (1913). The fate of protein digestion products in the body III. The absorption of amino acids from the blood by the tissues. J. Biol. Chem. 16, 197-212.

Vidaver, G.A. (1964). Transport of glycine by pigeon red cells. Biochem. 3, 662-667.

Vidaver, G.A. (1971). Glycine transport by pigeon red cells: calculation of glycine accumulation ratio by numerical integration of entry and exit rate equations. Biochim. Biophys. Acta 233, 231-234.

Villamil, M.F., Petori, V., Barajoss, L. and Kleeman, C.R. (1968). ECS and ionic distribution in the isolated arterial wall. Am. J. Physiol. 214, 1104-1112.

Wang, C.H. and Willis, D.L. (1965). Radiotracer Methodology in Biological Science. Englewood Cliffs, N.J., Prentice-Hall.

Webb, J.L. (1966). Iodoacetate and Iodoacetamide. In Enzyme and Metabolic Inhibitors, Academic Press, Vol. III, p. 1-283.

Weiss, G.B. (1966). Homogeneity of extracellular space measurement in smooth muscle. Am. J. Physiol. 210, 771-776.

Wheeler, K.P., and Christensen, H.N. (1967). Role of Na^+ in the transport of amino acids in rabbit red cells. J. Biol. Chem. 242, 1450-1457.

Wheeler, K.P., Inui, Y., Hollenberg, P.F., Eavenson, E. and Christensen, H.N. (1965). Relation of amino acid transport to sodium-ion concentration. Biochim. Biophys. Acta 109, 620-622.

Whittam, R. (1956). The permeability of kidney cortex to chloride. J. Physiol. 131, 542-554.

Wilson, T.H. (1962). Intestinal Absorption, W. B. Saunders Co., Phil., Chapter 5.

Wilson, T.H. and Wiseman, G. (1954). The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. J. Physiol. 123, 116-125.

Wiseman, G. (1951). Active stereochemically selective absorption of amino acids from rat small intestine. J. Physiol. 114, 7-8P.

Wiseman, G. (1953). Absorption of amino acids using an in vitro technique. J. Physiol. 120, 63-72.

Wolff, J. (1960). Thyroidal iodine transport I. Cardiac glycoside and the role of potassium. Biochim. Biophys. Acta 38, 316-324.

Wolowyk, M.W., Kidwai, A.M. and Daniel, E.E. (1971). Sodium-potassium stimulated adenosine triphosphatase of vascular smooth muscle. Can. J. Biochem. 49, 376-384.

Yamanchi, A. and Burnstock, G. (1969). Post natal development of smooth muscle cells in the mouse was deferens. A fine structural study. J. Anat. 104, 1-15.

Yunis, A.A., Arimura, ..and Kipnis, D.M. (1962). Amino acid transport and the sodium-potassium pump in human leukocytes. J. Lab. Clin. Med. 60, 1028.

Yunis, A.A., Arimura, G.K. and Kipnis, D.M. (1963). Amino acid transport in blood cells I. Effect of cations on amino acid transport in human leukocytes. J. Lab. Clin. Med. 62, 465-476.