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THE ASSOCIATION OF SODIUM AND POTASSIUM IONS WITH THE TRANSPORT OF NEUTRAL AMINO ACIDS IN THE EHRLICH ASCITES TUMOR CELL

by

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ABSTRACT

Chairman: John A. Jacquez

Within the past twenty years the transports of several non-electrolytes in several different in vitro and in vivo systems have been shown to require the presence of Na+ in the extracellular medium. Most of these systems show an additional sensitivity to the extracellular K+ concentration. Some current hypotheses propose that Na+ or K+ or both are bound to a second site on the non-electrolyte carrier, and that as a consequence of this binding the energy in either or both the Na+ and K+ concentration gradients is made available to the active transport of the non-electrolyte. The applicability of these hypotheses to the transport of neutral amino acids in the Ehrlich ascites tumor cell was examined in this study by measuring the changes in Na+ and K+ fluxes occurring during the uptake of alpha-aminoisobutyric acid (a non-metabolizable amino acid, the uptake of which shows a marked sensitivity to the Na+ concentration) and L-phenylalanine (an amino acid, the uptake of which shows little sensitivity to monovalent cations). In addition, the time course of uptake of alpha-aminoisobutyric acid (AIB) was examined during periods in which the Na+ gradient was reversed, and during periods in which both the Na+ and K+ gradients were reversed.

During the uptake of AIB the following changes in ion fluxes were observed: (1) An increase in the influx of Na+ (measured by the rate of ^{22}Na uptake), (2) An increase in Na+ efflux (calculated from Na+ influx and net flux data), (3) An increase in K+ efflux but no change in K+ influx (both measured by ^{42}K).

In these experiments the AIB uptake was also measured using tritium label, and it was found that the increase in Na+ influx was equal

to the saturable (sodium-dependent) portion of the AIB influx under a wide variety of conditions. Furthermore, both of these quantities were approximately equal to the sum of the increased efflux of Na+ and the increased efflux of K+. L-phenylalanine produced no significant changes in the one-way fluxes of either Na+ or K+.

Time studies of AIB uptake were then done using suspensions in which intracellular Na+ had been elevated by cold treatment and ouabain (2.5×10^{-4} M), and in which the extracellular Na+ was partially replaced by choline or K+. It was found that AIB could be actively accumulated while the Na+ gradient or both the Na+ and K+ gradients were reversed.

Other time studies of AIB uptake were conducted in media in which Na+ was replaced by K+, Li+, Cs+, Rb+ or choline. It was found that none of these media could support the rate of AIB uptake seen in a Na+ medium. However, Li+ did show a slight ability to substitute for Na+.

The results were interpreted to show that Na+ is bound to the Na+-sensitive amino acid carrier in a 1:1 stoichiometry with the amino acid at the extracellular side of the membrane. At the intracellular side the amino acid is released, and the Na+ is partially replaced by K+. However, in spite of the evidence showing ion movements on this carrier, it was demonstrated that neither the energy in the Na+ gradient nor the energy in both the Na+ and K+ gradients could account for the AIB gradient established. In view of this the transport process must have some other or some additional linkage to cellular metabolism.

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INTRODUCTION

In view of the prevailing hypothesis that life arose in the sea, and the fact that all multicellular organisms maintain an internal environment containing closely regulated concentrations of various ions (Smith, 1961), it is not surprising that many vital processes depend on the presence of ions. From ancient times until the early nineteenth century the need for salt in the diet was recognized, but the basis for this need was uncertain. With the coming of the modern scientific era, body ion content was first related to a specific disease by O'Shaughnessy (1831) who showed that cholera patients had reduced blood sodium and potassium concentrations. It was not until fifty years later that Ringer (1881) showed that the survival of tissues in vitro depended on the presence of several cations in prescribed concentrations, whereas the effect of anions was much less marked. These findings were confirmed by Overton (1910) who further speculated that cations might directly influence discrete reactions in cellular metabolism. One of the many points where this influence was subsequently demonstrated was the transport of sugars across the intestinal mucosa, a process which has been shown to reflect active transport across a cellular membrane (Crane, 1965).

In the early part of this century several investigators reported that one of the consequences of adrenalectomy was an impairment of glucose absorption from the intestine (Cori and Cori, 1927; Wilbrandt and Lengyel, 1933). The later work of Althausen et al. (1939) and Clark and MacKay (1942) showed that this deficiency could be relieved if the adrenalectomized animals were maintained on a high salt diet prior to testing the glucose absorption. These findings implied that the impairment of the glucose absorption was secondary to the cation imbalance observed in adrenalectomy. This hypothesis was confirmed by experiments on the in vitro gut preparation by Riklis and Quastel (1958). These investigators found that replacement of the sodium in the bathing medium with other cations severely reduced the absorption of glucose.

However, the in vitro gut preparation did not provide the first direct evidence for a cation effect on a transport process. Christensen and his coworkers (1952a) had previously demonstrated that the transport of amino acids by suspensions of the Ehrlich ascites tumor cell was affected by changes in extracellular sodium and potassium concentrations.

A. Amino Acid Transport in the Ehrlich Mouse Ascites Tumor Cell.

The Ehrlich mouse ascites tumor cell was developed about thirty-five years ago when a line of the Ehrlich mouse mammary carcinoma was converted to a free cell suspension capable of growth in the ascitic fluid of a host mouse (Loewenthal and Jahn, 1932). Since it was freely suspended and thus allowed the convenient alteration of the extracellular medium by centrifugation and resuspension, this cell has been widely used in biochemical and transport studies. Unlike other mammalian cell suspensions (such as erythrocytes or leucocytes), the Ehrlich ascites cell possessed the capability of both glycolytic and respiratory metabolism, and it showed a strongly concentrative uptake of many metabolic substrates.

Christensen and Henderson (1952) were the first to demonstrate that the Ehrlich cell could actively transport amino acids. They observed that the cells took up glycine and L-alanine in vivo to an intracellular concentration higher than the concentration in the ascitic fluid. Christensen and Riggs (1952) worked with the same suspension in vitro and obtained a strong concentrative uptake of several amino acids. They found that the cell could produce a concentration difference across the cell membrane of up to 60 mM for glycine. Subsequent research established that the intracellular amino acid was in free solution rather than bound within the cell. Christensen and Riggs (1952) observed that the cells swelled with the uptake of amino acids, which indicated that the latter were osmotically active inside the cells. Christensen and Riggs (1952) also observed that several inhibitors of respiration and glycolysis could prevent active accumulation. This led them to state that the process

was linked in some manner to cellular metabolism. More extensive inhibitor studies were later done by Johnstone and Scholefield (1959) and gave the same results. Heinz (1957) demonstrated that the effect of inhibitors was to decrease the influx of the amino acid but not to increase its efflux; the latter would have been expected if the inhibitor were acting to decrease the number of intracellular binding sites.

Heinz (1954) has shown that the flux measured over the first 2 min has a striking dependence on temperature with Q_{10}'s in the range of 3.0 to 4.0. In this same study Heinz also examined the kinetics of amino acid uptake, and demonstrated that the flux coefficient (i.e., the rate of uptake divided by the extracellular concentration) decreased as the extracellular concentration of the amino acid increased, i.e., the uptake of the amino acid exhibited saturation with increasing extracellular concentration. Heinz was able to show that the curve relating uptake to extracellular concentration had two components: a linear increase in uptake with increasing extracellular concentration, presumed to represent the contribution of diffusion; and a saturable component which had a maximal rate of operation. Heinz then examined the saturable component and found that it could be adequately described by the Michaelis-Menten equation of enzyme kinetics. In accord with this formulation, he further demonstrated that plots of the inverse of the initial amino acid uptake rate were linear with respect to the inverse of the amino acid concentration. As in other transport systems this finding has been the most important factor in the formulation of a carrier theory of membrane transport as described by Wilbrandt and Rosenberg (1961). According to this theory, the substrate combines extracellularly with a molecule within the membrane (the carrier) which mediates substrate movement through the membrane and releases it intracellularly. Analogy of the carrier to an enzyme is obvious considering that the carrier itself is assumed not to be permanently modified by the translocation, and that carriers usually exhibit considerable specificity for the substrate. Furthermore, all of the phenomena observed in transport can be explained in terms of the

carrier hypothesis.

As would be expected if a selective combination site were involved in transport, the structure of the amino acid has a great effect on its rate of uptake in the Ehrlich ascites cell. For example, although no difference in membrane "solubility" would be expected, L-amino acids enter faster than their D stereoisomers (Christensen et al., 1952a, Oxender, 1965), beta amino acids are concentrated to a lesser degree than alpha amino acids, and gamma amino acids are not concentrated at all (Christensen et al., 1962).

Also in analogy with enzyme kinetics, Christensen et al. (1952a) have shown that the uptake of a given amino acid is decreased by the presence in the extracellular medium of a second amino acid as would be expected if the two amino acids were competing for combination with the same carrier site. Paine and Heinz (1960) strengthened this interpretation by demonstrating that the presence of the second amino acid increased the apparent Michaelis constant (K_m) rather than decreasing the maximal uptake velocity (v_{max}) of the first amino acid, the same result as is found in enzyme kinetics.

Perhaps the best support for a mobile carrier hypothesis was presented by Heinz (1954, 1957) who observed that preloading Ehrlich cells with unlabeled glycine increased the initial influx of labeled glycine. Furthermore, reversal of the label asymmetry showed that the extracellular amino acid increased the efflux rate of the intracellular label. This phenomenon was interpreted as a case of flow driving counterflow or exchange diffusion by means of a mobile carrier (Wilbrandt and Rosenberg, 1961). Jacquez (1961b, 1963a, 1963b) and Johnstone and Scholefield (1961) have also provided examples of a given preloaded amino acid increasing the influx of a different extracellular amino acid, i.e., heteroexchange. Examination of the kinetics of exchange diffusion has shown that the intracellular amino acid behaves as a kinetically uniform pool (Heinz, 1957). This is good evidence against the possibility of a binding process which is either irreversible or which has low rate constants.

One of the most active areas in the study of amino acid transport in the Ehrlich ascites cell has been the investigation, primarily by Christensen and his coworkers, of evidence for the existence of several distinct amino acid carriers. Christensen (1955a) originally proposed at least three different carrier systems: one for the uptake of neutral, one for cationic and one for anionic amino acids. This hypothesis resulted from the observation that members of any one group failed to inhibit the uptake of members of the other two groups (Christensen et al., 1952a). Similar evidence was then used to support the postulation of several different carriers for the neutral amino acids (Tennenhouse and Quastel, 1960; Johnstone and Scholefield, 1961; Oxender and Christensen, 1963). The suggestion of further carrier multiplicity has come from studies on beta amino acids (Christensen, 1964a), diamino acids (Christensen, 1964b; Christensen and Liang, 1966a), and the nonsaturable uptake of certain amino acids (Christensen and Liang, 1966c).

On the other hand, Jacquez (1961a, 1963b, 1964) has conducted extensive mathematical analyses on some simple models of carrier operation, and has found that a wide spectrum of competitive interactions could be explained with only a single carrier, thereby obviating the hypothesis of carrier multiplicity merely on the basis of competition behavior. Nevertheless, Oxender and Christensen (1963) have provided evidence to establish the existence of at least two carriers for the neutral amino acids. One carrier was proposed to mediate primarily the uptake of amino acids with polar side chains (e.g., threonine, serine, proline), but it was best characterized by the uptake of alanine and was termed the alanine-preferring or A-system. The other carrier apparently mediates the uptake of longer hydrocarbon side chain or aromatic amino acids (e.g., leucine, isoleucine, valine and phenylalanine). This system was best characterized by the uptake of leucine and was labeled the leucine-preferring or L-stystem. These systems were differentiated not only by the relatively poor competition between the A and L type amino acids but several other factors as well. The L-system amino acids

showed faster rates of uptake but low steady-state distribution ratios, whereas the A-system amino acids exhibited the reverse. The L-system was shown to exhibit exchange diffusion to a greater extent than the A-system. Furthermore, the A-system was far more sensitive to metabolic inhibitors, anoxia, pH, extracellular cation concentrations, and had a higher temperature coefficient. As will be discussed later, it was the differential sensitivity of the A and L-systems to extracellular cation concentrations that provided the conclusive proof for the existence of the duality.

The demonstration of two separate systems for the transport of neutral amino acids has also provided a workable explanation for the most unique phenomenon of amino acid transport in the Ehrlich ascites cell, competitive stimulation. Jacquez (1961b), while measuring tryptophan uptake, observed that its accumulation could be enhanced both in rate and steady-state level by the simultaneous presence of several other amino acids, whereas competition between the two amino acids would have been expected. Subsequently, Chirigos et al. (1962) found that in the Sarcoma 37 ascitic tumor the uptake of tyrosine was increased by the amino acid analog p-fluorophenylalanine. Oxender and Christensen (1963) felt that this phenomenon could be explained by the following hypothesis: if the amino acid producing stimulation had good affinity for the A and for the L carriers it would be accumulated by the A-system and drive an increased uptake of the other amino acid by counterflow on the exchanging L carrier. The recent work of Jacquez (1967) and Schafer and Jacquez (1967a) has provided strong evidence in favor of this explanation. Although the phenomenon was initially unique among transport systems, Munck (1966) subsequently demonstrated it in the intestinal transport of amino acids.

B. The Effects of Sodium and Potassium on Amino Acid Transport in the Ehrlich Ascites Cell.

Since the earliest studies on the in vitro uptake of amino acids by

the Ehrlich ascites cell, the importance of cations has been recognized. Christensen and Riggs (1952) observed that amino acid distribution ratios were decreased when extracellular potassium was increased above 40 mM. They also observed there was a loss of intracellular K+ and a gain of Na+ during the period of amino acid accumulation. Christensen et al. (1952a) corroborated these findings and added that a decrease in extracellular K+ below 4 mM also inhibited amino acid uptake. They also observed a substantial decrease in the uptake of amino acids when one-half of the extracellular sodium was replaced by choline. Christensen et al. (1952b) reported that almost all the intracellular potassium was displaced by the accumulation of α,γ-diaminobutyric acid; however, since this amino acid is cationic such a displacement might have been secondary to the electrogenic effect of the accumulation. The magnitude of the ion shifts observed in the early studies is certainly open to question since all incubations were of long duration (1-2hr), and a net increase in sodium and decrease in potassium might have been expected due to deterioration of the cells.

Riggs et al. (1953, 1954) further observed that certain compounds which increased amino acid uptake, e.g., pyridoxal and indolacetate, also increased the efflux of K+. Christensen (1955b), upon noting that pyridoxal could complex certain cations, postulated that pyridoxal was part of the carrier mechanism. Riggs and Christensen (1956) strengthened the evidence for an association of potassium, amino acid and pyridoxal in transport with the observation that stimulation of amino acid transport by pyridoxal required the simultaneous presence of extracellular potassium. They also demonstrated that lowering extracellular potassium to less than 4 mM caused a concomitant loss of K+ from the cells. They interpreted this K+ loss as the ultimate cause of the decreased amino acid uptake observed at low K+ levels. All of the above findings linking K+ to the amino acid transport system led Christensen (1955a) to compare the effect of K+ to that of a competing amino acid. Riggs et al. (1958) acted on this hypothesis by examining the effects of

intracellular potassium on amino acid accumulation. To do this they depleted cells of potassium to various extents by treatment with DNP and with ion exchange resins. They found that the resulting decrease in amino acid transport was directly proportional to the decrease produced in intracellular K+. However, these investigators failed to take into account the fact that the intracellular K+ loss was mirrored by a sodium gain. Nevertheless, in the light of their results, Riggs and his coworkers proposed what has come to be known as the potassium gradient hypothesis of amino acid transport. This hypothesis supposed that the amino acid carrier site also had an affinity for potassium, thus amino acid and potassium competed for this site. The concentrative uptake of any amino acid could have been accounted for by a counterflow of potassium out of the cell via the carrier. Therefore, the potassium ion gradient produced by the operation of the ion pump would have been the ultimate source of energy for the accumulation of amino acids. Heinz and Patlak (1960) gave an excellent theoretical discussion of the free energy changes that would be expected in the active transport and exchange diffusion of amino acids. They also computed the osmotic energy available in the K+ gradient and found it corresponded closely to the free energy requirement of active amino acid transport. However, explanation of the amino acid transport by a counterflow of potassium would have required a coupling of the two gradients with the improbable efficiency of approximately 100%. Mitchell (1962) has also discussed the feasibility of coupling a gradient in one substrate to that of another substrate.

The K+ gradient hypothesis was soon discredited by the work of several investigators, but the seeds of its destruction actually lay in the earlier work of its proponents. Christensen et al. (1952a) had depleted cells of potassium and found that the glycine distribution ratio decreased only slightly as intracellular K+ fell from 108 to 52 mM. Furthermore, in their experiments (Riggs et al., 1958; Christensen, 1955a) which indicated that amino acid transport decreased as extracellular potassium increased, the increase in extracellular K+ was always complemented by

a decrease in extracellular Na+. Kromphardt et al. (1963) later showed that it was actually this decrease in extracellular sodium that was responsible for the decreased amino acid uptake. To do this they substituted choline for either sodium or potassium extracellularly. Thus, either sodium or potassium could be varied independently while maintaining osmolarity with the choline. The results of this study proved that amino acid transport did not decrease as extracellular potassium increased if the extracellular sodium concentration were held constant; on the other hand, there was a very definite decrease in amino acid transport when the Na+ concentration was decreased in the presence of a constant extracellular K+ concentration.

Also, the earlier studies of Heinz (1961) had shown that there was no change in potassium efflux as the intracellular concentration of glycine increased although a decrease would have been expected if potassium were competing for the amino acid site. Wong (1965), however, has demonstrated by a theoretical analysis that coupling of potassium efflux to amino acid entry could still occur without competition between the two if a two-site carrier were proposed. The final elimination of the potassium gradient hypothesis has come from the work of Hempling and Hare (1961) who measured the one-way fluxes of potassium and glycine during glycine uptake. These investigators also computed the minimal rate of expenditure of free energy necessary for the maintenance of the glycine gradient and the maximal rate of expenditure of free energy from the K+ gradient by measuring the steady-state distribution ratios and fluxes of both. They have stated that the K+ gradient could not drive the accumulation of glycine for the following reasons: (1) Glycine increased both the influx and efflux of K+ whereas the hypothesis would predict only an increase in efflux. (2) The apparent affinity of K+ for the carrier was much less than that of glycine both intracellularly and extracellularly. (3) The minimal rate of expenditure of free energy necessary for the maintenance of the glycine steady-state distribution ratio was 30% greater than the maximal rate of expenditure of free energy from the K+ gradient.

The association of K+ and amino acid transport was made more unlikely by experiments indicating that pyridoxal did not have a specific function in amino acid transport. Christensen (1960) and Oxender and Royer (1961) have shown that pyridoxal increased the accumulation of amino acids by decreasing their efflux rather than increasing their influx. Furthermore, Hempling (1961) and Hempling and Hare (1965) have demonstrated that, under conditions in which there was a net K+ influx, the presence of glycine and pyridoxal actually decreased influx, efflux and net flux of K+. However, the most important information arising from the controversy over the K+ gradient hypothesis was the demonstration of Kromphardt and his coworkers (1963) that amino acid transport in the Ehrlich ascites cell was most sensitive to sodium not potassium extracellular concentration. This laid the ground work for the later work relating amino acid uptake to the sodium ion gradient. However, in this regard, the work of several investigators on the involvement of Na+ in transport systems in the intestinal mucosa preceded that in the Ehrlich ascites cell.

C. The Sodium Dependent Absorption of Sugars in the Intestine.

Höber (1899) gave the first evidence that the intestinal absorption of sugars was mediated by a specific transport system. Barany and Sperber (1939) proposed that glucose was actively transported across the mucosal wall. However, due to the rapid metabolism of glucose by the intestinal cells, an unambiguous demonstration of active sugar transport was not in evidence until Campbell and Davson (1948) demonstrated the active absorption of the non-metabolizable sugar analog, 3-0-methylglucose. Since that time sugar transport in the intestine has been well-characterized and shown to exhibit the expected phenomena of a carrier mediated process. This development has been well discussed in the comprehensive review of Crane (1960).

As stated before, Riklis and Quastel (1958) provided the first in vitro evidence (using isolated guinea pig small intestine) that sugar

transport in the gut depended on the presence of Na+; however, they also stated that K+ or rubidium also stimulated sugar uptake, a finding that has not been corroborated. Their findings on the Na+ requirement for sugar transport were confirmed by Csaky and Thale (1960) who showed that 3-0-methylglucose absorption by the toad gut in vitro was abolished by substituting Li+, Mg++ or choline for Na+ in the mucosal medium. Csaky and Zollicoffer (1960) obtained the same results on isolated segments of the small intestine of the rat in situ, and they also found that the active transport process could be reestablished by returning Na+ to the medium, thereby demonstrating the effect was reversible. Csaky et al. (1961) found that ouabain and thevetin at 10^{-7} M inhibited sugar transport. They interpreted this to indicate a linkage of the sugar and ion transport systems. However, since the gut they used had been incubated with the ouabain for a period of six hours or more, it is probable that the tissue had deteriorated, and that the ion gradients had for the most part disappeared. Bihler and Crane (1961, 1962) unsuccessfully tried to substitute several other alkali metal cations, tris, choline and mannitol for sodium. They also showed that the rate of transport, steady-state levels and exchange rates all depended almost linearly on the extracellular sodium concentration. Bihler et al. (1962) then examined the absorption of sugar under aerobic and anaerobic conditions, and they found that both the energy-dependent (aerobic) active transport process and the facilitated diffusion process (anaerobic) depended on the presence of Na+ in the mucosal medium. This finding led them to propose that Na+ was actually combining with the sugar carrier. Crane et al. (1961), while investigating the effects of ouabain on sugar absorption, showed that it inhibited the active transport of sugars but not the facilitated diffusion uptake. Facilitated diffusion did, however, require the presence of sodium. Crane and his coworkers then suggested a dual effect of sodium: first, that sodium moved with the sugar carrier, and secondly, that a sodium gradient maintained by the sodium pump was necessary for active sugar transport. These observations led Crane

(1962) to propose a two-site carrier. Crane's model hypothesized that the carrier had a site for sugar attachment and a site for ion attachment and that the latter site required the presence of sodium for movement of the carrier complex to occur. Crane predicted that the asymmetry in the sodium gradient could drive the uptake of the sugar, a situation analagous to the K+ gradient proposal. He felt that such a linkage of the energy established by the ion pump to the sugar uptake would be of economic advantage to cellular metabolism. Crane (1964) provided a good deal of support for his hypothesis in a preliminary report in which he reported that sections of gut, preloaded with sugar and then placed in a sodium-free medium, exhibited an active efflux of the sugar. In other words, reversal of the sodium gradient produced efflux of the accumulated sugar to the extent that its extracellular concentration exceeded the intracellular concentration. However, this experiment was conducted under a situation of complete metabolic inhibition of the tissue by dinitrocresol and anoxia.

The best support for the sodium gradient hypothesis came from the studies of Barry and those of Schultz and Zalusky on changes in the transmural potential and short-circuit current across the gut during sugar accumulation. Barry et al. (1961), while measuring the transmural potential developed by isolated segments of rat small intestine, noted that the addition of glucose to the mucosal medium increased the observed potential difference. Furthermore, this increase could be prevented by the simultaneous presence of phlorizin, a competitive inhibitor of sugar uptake. Their interpretation was that sugar entry into this tissue promoted an additional sodium entry. This interpretation was supported by the finding of Bosackova (1963) that 6-deoxyglucose uptake in the gut produced an increased influx of Na+ as measured by ^{22}Na. Schultz and Zalusky (1963a, b) later confirmed the findings of Barry and his coworkers, and they domonstrated that the short-circuit current was also increased by the presence of glucose in the mucosal medium. These authors also established that the short-circuit current changes

reflected changes in the transmural potential difference. Furthermore, by using ^{24}Na to measure one-way fluxes in the rat gut, Schultz and Zalusky (1963b) proved that the short-circuit current was equal to the net Na+ influx, thus directly establishing the relationship of short-circuit current to sugar and sodium movements. Later studies (Schultz and Zalusky, 1964 a) showed that ouabain could prevent the change in potential difference. Schultz and Zalusky (1964 b) also demonstrated that the increase in short-circuit current could be eliminated by phlorizin. More importantly, they measured the change in potential difference as a function of the mucosal glucose concentration, and found that it exhibited a plateau at high sugar concentrations, implicating a saturation phenomenon. Schultz and Zalusky suggested that this finding could be explained by a 1:1 stoichiometry of sodium and glucose on the sugar carrier. Asano (1964) also reported a saturation behavior of the increase in short-circuit current as the glucose concentration was increased. The association of the observed potential difference change and the short-circuit current change with the sodium gradient hypothesis of Crane has been further discussed by Barry et al. (1964) and Curran (1965). Bosackova and Crane (1965a) completed the evidence for an association of sodium and glucose movements by the observation that sodium absorption (measured by ^{22}Na) increased during sugar transport, although they attempted no stoichiometric analysis.

The next important contribution to the sodium gradient hypothesis came from the analysis of the kinetics of sugar transport. By measuring the uptake of 6-deoxyglucose at various mucosal concentrations of the sugar and Na+, Crane et al. (1965) found that as the extracellular Na+ concentration rose, the apparent Km of the carrier for the sugar decreased, i.e., Na+ appeared to increase the affinity of the carrier for the sugar. This introduced a second asymmetry into the carrier hypothesis, and Crane and his coworkers modified their sodium gradient hypothesis in the light of this finding. They proposed that at the mucosal side of the brush border membrane the high concentration of Na+

saturated the carrier sodium sites, consequently increasing the carrier's sugar affinity and permitting translocation of the carrier complex to the intracellular side of the membrane. At the intracellular side of the membrane sodium would tend to dissociate from the carrier due to the lower intracellular sodium concentration, thereby reducing the affinity of the carrier for sugar. Thus, the carrier would release sugar and sodium intracellularly and would return, supposedly by diffusion, to the extracellular side of the membrane.

The finding of a sodium effect on the sugar K_m has been supported by the observations of Lyon and Crane (1966a, b). They also observed that the mucosal sodium concentration had no effect on the v_{max} of sugar transport in the rat small intestine preparation. In contrast, Schultz and Zalusky (1964b) have reported that the Na+ concentration had no effect on the K_m for sugar transport in the rabbit small intestine preparation, but had rather a marked effect on the v_{max}. They found that v_{max} decreased as the extracellular sodium decreased. Lyon and Crane (1966a) proposed that this discrepancy might result from a species difference, and they also noted that in their experiments sodium had been replaced by Tris, whereas in the experiments of Schultz and Zalusky sodium had been replaced by potassium.

Additional support of the sodium gradient hypothesis came from Lyon and Crane (1966b, c) who showed that plots of the inverse of the change in the potential difference or the inverse sugar uptake against the inverse of the sodium concentration were linear. Semenza (1967) has developed the rate equations for enzyme systems with substrate and and activator, and has applied them to data on sugar transport in the gut. He thereby demonstrated that the linearity observed by Lyon and Crane would be expected if and only if the sugar and Na+ were combining with the carrier in a 1:1 stoichiometry. Bihler et al. (1967) also observed that, in the transport of sugars which exhibit facilitated diffusion but not active transport, the K_m was not affected by the extracellular sodium concentration although the rate of equilibration was dependent upon the

presence of Na+. They interpreted this to indicate that the alteration of sugar K_m by Na+ was the crucial factor in the production of a sugar concentration gradient.

The primary opposition to the sodium gradient hypothesis of Crane arose from the findings concerning the effect of K+ on the transport system. Csaky and his coworkers have repeatedly shown that substitution of K+ for Na+ in the mucosal medium has a greater inhibiting effect on sugar transport than the substitution of Mg++, choline, or Tris (Csaky 1961a, 1961b, 1963; Csaky and Zollicoffer, 1960). Bosackova and Crane (1965b) confirmed this observation by demonstrating that replacement of Na+ by K+ was more inhibitory than replacement by Li+. They interpreted this to indicate that K+ competed with Na+ for combination on the carrier ion site, and they have subsequently incorporated this effect into the sodium gradient hypothesis. Since the finding of the K+ effect, Crane (1965) has proposed a revised version of the sodium gradient hypothesis which takes into account the role of the potassium ion. According to this theory, the sodium ion and its gradient operated in exactly the manner proposed in their earlier sodium gradient hypothesis. However, Crane also proposed that when the carrier was on the intracellular side of the membrane, potassium displaced sodium from the ion site, and thereby raised the K_m of the carrier for the sugar. On the extracellular side of the membrane the K+ would dissociate from the carrier due to the lower K+ concentration there. An increase in extracellular K+ would be expected to interfere with sugar transport since the potassium would compete for the sodium site, and by doing so raise the effective K_m for the sugar. This revised model, which may be conveniently termed the sodium-potassium gradient hypothesis, was supported by additional findings from Crane's laboratory. Bosackova and Crane (1965a) demonstrated that K+ inhibited sugar and ^{22}Na influx to approximately the same extent, and that, at constant Na+ concentration, the K_m for the sugar increased as the extracellular potassium concentration increased. This result was confirmed by the observations of Lyon and

Crane (1966a, b) who observed that the K+ decreased the change in potential difference occurring during sugar transport.

Additional objections to Crane's theory came from the laboratory of Csaky. Csaky (1963) reported that 3-0-methylglucose could be concentrated even in the presence of a reversed sodium gradient. Crane (1965) stated, however, that this would be expected since Csaky used high enough concentrations of sugar to saturate the carrier in spite of the elevated K_m. However, Csaky and Hara (1965) have tested the effect of ouabain on sugar transport and have found it is inhibitory to the uptake even before the ion gradients have changed significantly. Thus Csaky still favors some connection of ion and sugar transport systems which might be related to a common energy supply as will be discussed later. It should also be noted that the sodium-potassium gradient hypothesis proposes that the sugar transport carrier operates with electroneutrality since sodium rides in with the sugar carrier, but potassium displaces the sodium and rides out with the carrier. This explains the observations of several investigators that non-penetrating anions such as sulphate do not inhibit sugar absorption (Crane, 1962; Csaky and Zollicoffer, 1960). However, Crane has not explained how such an electroneutral operation of the carrier can produce an increase in the potential difference across the membrane during sugar transport.

D. The Sodium Requirement of other Intestinal Transport Systems.

Sugar transport is not the only absorptive system in the gut that depends on mucosal sodium concentration. Nathans et al. (1960) gave the first evidence for the dependence of amino acid absorption in the gut on Na+ when they observed a decrease in L-monoiodotyrosine uptake upon replacing Na+ by each of several other cations. Csaky (1961a, b) observed the same for L-tyrosine transport. Harrison and Harrison (1963) also reported that K+ was inhibitory to amino acid uptake. Robinson and Felber (1965a) have demonstrated that both the facilitated diffusion (occurring during anoxia) and the active transport of amino acids

require the presence of Na+. Schultz and Zalusky (1965) have found an increased short-circuit current across the gut wall during amino acid transport. Furthermore, as in the case of sugar transport, this increased current shows saturation as the extracellular amino acid concentration is elevated. All of these results have been interpreted as demonstrating the same sodium-potassium gradient mechanism as for sugar transport (Schultz and Zalusky, 1965; Robinson and Felber, 1965b; Robinson, 1966). However, sugar and amino acid transport cannot be mediated by the same carrier since Schultz and Zalusky (1965) have shown that the addition of alanine in a saturating concentration gave a maximal increase in short-circuit current; however, when 3-0-methylglucose was then added in addition to the alanine there was a further increase in the transmembrane potential. Other preliminary results also indicate that the transport of arbutin (Alvarado, 1965) and the transport of bile salts (Holt, 1964; Playoust and Isselbacher, 1964) depend on the presence of Na+ and are inhibited by the presence of K+. Furthermore, Lyon (1967) has shown that bile salts produce an increase in the potential difference across the intestinal wall when added to the mucosal medium.

E. The Sodium Gradient Hypothesis in Other Systems.

Additional support of Crane's basic sodium gradient hypothesis has come from studies on the avian red blood cell and the mammalian reticulocyte. Christensen et al. (1952c) were the first to show a concentrative, saturable uptake of amino acids in the avian erythrocyte. These authors also stated that this uptake was highly dependent on the presence of extracellular Na+, but that it was not sensitive to most respiratory inhibitors or fluoride. Vidaver et al. (1964) extended these early observations using the pigeon red blood cell to show that the K_m, not the v_{max}, of amino acid uptake depended on sodium. Furthermore, plots of the inverse of the amino acid uptake against the inverse square of the sodium extracellular concentration were shown to be linear. Vidaver suggested that this was due to a combination of sodium on the amino acid carrier in a 2:1 stoichiometry. Vidaver (1964a), in a further set

of experiments, demonstrated that: (1) neither choline, K+ nor Li+ could substitute for Na+, (2) the exit of glycine depended on intracellular sodium concentration and (3) extracellular K+ had little effect on glycine uptake if Na+ were held constant. Vidaver (1964b, c) then turned his attention to the sodium gradient across the cell membrane. He hemolyzed and restored cells in a sodium medium to obtain Na+-loaded red blood cells. He found that these cells, when preloaded with glycine and placed in a low Na+ medium, could establish an active efflux of the glycine. This was in complete accord with Crane's sodium gradient hypothesis. Finally, to confirm his suggestion that two sodium ions were entering the cell per amino acid transported, Vidaver (1964d) measured the influx of ^{22}Na during glycine uptake and found it to be approximately two times the glycine influx. Wheeler et al. (1965) have also noted that Augustinsson plots of glycine uptake in these cells depended on the square of the extracellular sodium for glycine levels above 20 mM; however, the dependence was on the first power of the sodium concentration for glycine levels below 20 mM. Wheeler and Christensen (1967a) have further shown that amino acid uptake in the pigeon red blood cell increases both sodium influx and efflux. Their calculated ratio of the increased sodium influx to amino acid influx ranges from 2.52 for alanine to 0.96 for β-alanine.

More recently Christensen's laboratory has reported amino acid uptake in rabbit reticulocytes exhibits many of the characteristics of that in the pigeon red blood cell (Winter and Christensen, 1965). Wheeler and Christensen (1967b) have examined the sodium dependence of this uptake system and feel that maturation of the reticulocyte is accompanied by a decreased sodium binding capacity of the amino acid carrier and a consequent loss of its concentrating ability. The same investigators, however, have also reversed the sodium gradient across these cells and report that extracellular amino acid was still accumulated to distribution ratios greater than 1.0. Moreover, they found a decrease in v_{max} and an increase in K_m occurred with decreased extracellular Na+.

This effect on v_{max} was not in concert with the sodium gradient hypothesis. Other evidence against the combination of sodium with the amino acid carrier has come from the work of Winter and Christensen (1965) on rabbit reticulocytes and Wheeler et al. (1965) on pigeon red blood cells. These investigators found no effect of sodium on the rate of exchange diffusion of amino acids, whereas the sodium gradient hypothesis, in its original form, demanded a combination of sodium with the carrier for any movement of the carrier-amino acid complex (Crane, 1965). Crane et al. (1965) have since suggested that the carrier is capable of translocating without Na+ on the ion site; however, in the absence of sodium the K_m of the carrier for amino acid would be correspondingly higher.

Recently, the sodium gradient hypothesis has also been examined with regard to the requirements of amino acid transport in the Ehrlich ascites cell. Wheeler et al. (1965) have reinterpreted the data of Riggs et al. (1958), and they suggest that amino acid transport in these experiments depended on the intracellular sodium not the intracellular potassium concentration. They have also shown that plots of the increase in the amino acid uptake against the inverse of the sodium ion concentration are linear, which suggested a 1:1 amino acid to sodium stoichiometry on the carrier. However, this group has also reported that these plots demonstrated both the K_m and the v_{max} of the transport process are altered by changes in the extracellular sodium concentrations. This was confirmed by the very complete analysis of Inui and Christensen (1966) who have also established quite clearly the existence of the sodium-dependent A carrier system and the sodium-independent L carrier system. The essential part of this work was the demonstration that the portion of methionine uptake (presumed to be mediated by both carriers) inhibitable by the removal of sodium is precisely the same as that portion inhibited by high concentrations of alpha-aminoisobutyric acid, the saturable uptake of which was shown to be completely sodium-dependent and thus mediated by the A carrier system.

An almost parallel development of sodium involvement has occurred with the study of amino acid transport in striated muscle. Kipnis and Parrish (1965) and Kipnis (1965) have shown that removal of extracellular sodium eliminated the concentrative uptake of amino acid but did not alter the facilitated diffusion of sugars into the muscle, and that the Na+ affected primarily the amino acid K_m, not the v_{max}. Furthermore, Kipnis and Parrish (1965) have shown the plot of the inverse of the amino acid uptake velocity against the inverse of the sodium ion concentration is linear, a fact which they also interpreted to indicate a 1:1 stoichiometry of amino acid and sodium. However, these investigators oppose an interpretation of their results in terms of the sodium gradient driving the uptake of amino acid, because they have found: (1) the uptake of amino acid decreased as extracellular sodium decreased even if the sodium gradient were the same; (2) ouabain did not affect the amino acid uptake even though it caused an increase in intracellular and a decrease in extracellular sodium. Parrish and Kipnis (1964) have suggested, along the same lines as Csaky, that the sodium effect is at some intermediate energy-linkage step in the transport process.

Studies of amino acid transport in kidney cortex slices have revealed a dependence on the concentration of sodium and an inhibitory action of ouabain (Burg and Orloff, 1962; Vogel and Krȍger, 1966). Furthermore, the transport process operates maximally only over a narrow range of potassium concentration (Fox et al., 1964).

Adamson et al. (1964, 1966) and Adamson and Anast (1966) have shown sodium dependence and a potassium sensitivity of amino acid transport into bone and embryonic cartilage. These workers also showed that ouabain could inhibit this amino acid accumulation. The same results have been established for the uptake of several amino acids into L-strain mouse fibroblasts (Kuchler and Marlowe-Kuchler, 1965; Kuchler, 1967) and leucocytes (Yunis et al., 1963). In addition, sodium dependence has been shown for: ascorbic acid entry into the adrenal

cortex (Sharma et al., 1964); quaternary ammonium compounds, serttonin and norepinepherine in the choroid plexus (Tochino and Schanker, 1965a, b); choline uptake in presynaptic terminals of cholinergic nerves (Birks, 1963); amino acid transport in brain cortex slices (Abadom and Scholefield, 1962; Takagaki et al., 1959); and amino acid entry into thymus cell nuclei (Allfrey et al., 1961) and subcellular brain particles (Weinstein et al., 1965). Although most bacteria grow well on media devoid of sodium, some marine bacteria apparently require sodium for amino acid transport (Drapeau et al., 1963; Stevenson, 1965). Furthermore, sodium ions appear to be necessary for certain hormone actions, e.g., the insulin stimulation of sugar uptake in adipose tissue (Kujalova and Mosinger, 1966) and the adrenalin stimulated release of fatty acids in adipose tissue (Mosinger and Kujalova, 1966).

This widespread occurrence of the sodium requirement has been used by Crane (1965) to suggest a possible universal mechanism whereby ion gradients could supply the necessary energy for the active accumulation of non-electrolytes. Or, in reverse, the presence of certain non-electrolytes could cause "downhill" fluxes of sodium and potassium, a phenomenon that could account for synaptic mediation by acetylcholine or gamma-aminoisobutyric acid. Nevertheless, there are several investigators who feel that the sodium ion requirement is not for combination with the substrate carrier but is at some step in the linkage of cellular metabolism to the transport process as suggested by Csaky (1963). Foremost among the possibilities of such a site of action is the membrane adenosine triphosphatase.

F. The Na+-K+ Sensitive Adenosine Triphosphatase.

The occurrence of a cell membrane ATPase was first reported by Skou (1957) who isolated the enzyme from crab peripheral nerves. He has shown that this ATPase is activated by Mg++ and inhibited by Ca++. Action of the enzyme requires Na+ but is further increased by low concentrations of K+. It is the potassium requirement that has

provided the main analogy of the ATPase to the sodium-sensitive transport processes (Kleinzeller and Kotyk, 1961). In analogy with several of the transport processes discussed, Skou has shown that the ATPase is inhibited both by complete elimination of K+ and by elevation of the K+ above a certain low level. Post et al. (1960) subsequently hypothesized that the ATPase was a part of the ion pump mechanism, and Charnock et al. (1966) have localized the enzyme in the cell membrane by electron microscope autoradiography. Furthermore, membrane ATPases have been demonstrated to be present in the Ehrlich ascites cell (Wallach and Ullrey, 1964) and in the intestinal brush border membrane (Skou, 1965).

Maio and Rickenberg (1962) were the first to suggest that a membrane ATPase might be involved in amino acid and sugar transport when they demonstrated that in L-strain tissue culture cells both uptakes were sodium-dependent and yet were mediated by separate carriers. Csaky (1963) also felt the requirement of sodium for both amino acid and sugar transport in the gut indicated that the sodium was required at some metabolic step common to all transport processes. The hypothesis was supported by investigations on the effect of ouabain, a specific inhibitor of the ATPase. Bittner and Heinz (1963) observed that ouabain decreased glycine uptake by the Ehrlich ascites cell before intracellular K+ had fallen significantly, and they felt this indicated the direct involvement of a membrane ATPase.

Observations on exchange diffusion in the Ehrlich ascites cell have also provided some indirect supporting evidence. Jacquez and Sherman (1965) have shown that metabolic inhibition does not alter the rate of exchange diffusion of several amino acids. Johnstone and Scholefield (1965) have reported that Na+ is not required for the homoexchange of methionine, whereas it is required for the active transport of methionine. Combining the results of these two studies would suggest that the sodium ion is not necessary for the translocation of the carrier (occurring in exchange diffusion) but rather for the coupling of metabolic energy to this carrier (active transport).

G. Questions Posed and Summary of Results.

As is shown by the above review, the involvement of sodium and potassium in non-electrolyte transport has raised several questions. These questions are related primarily to the site of action of the cations in the transport process and the possible coupling of the energy in the sodium, or in the potassium gradient, or in both to the active transport of non-electrolytes. I have attempted to answer some of these questions for the sodium- and potassium-influenced transport of amino acids in the Ehrlich ascites tumor cell. Since there is a definite dichotomy of neutral amino acid uptake in the Ehrlich cell, I have employed two amino acids: alpha-aminoisobutyric acid (AIB), which is concentrated almost exclusively by the sodium-dependent A-carrier system, (Inui and Christensen, 1966); and L-phenylalanine, which is taken up primarily by the Na+-independent L-carrier system (Oxender and Christensen, 1963). The most relevant questions considered and a brief summary of my results concerning each are presented here.

1) Does sodium actually move on the amino acid carrier, and if so, in what stoichiometry? I have observed that an increased influx of sodium (measured by ^{22}Na) does occur during the transport of AIB. Furthermore, this increase in Na+ influx was the same as the saturable portion of the AIB influx under a wide variety of conditions. No increase in Na+ influx was found during the transport of L-phenylalanine. These results suggest that sodium is moving on the A-system carrier, but not the L-system carrier, in a 1:1 stoichiometry with the amino acid. A preliminary report of these results has been published (Schafer and Jacquez, 1967b).

2) Is there an increased efflux of potassium during amino acid uptake as would be suggested by the sodium-potassium gradient hypothesis of Crane? I have measured the efflux of K+ (using ^{42}K) during the accumulation of AIB and L-phenylalanine and have found it to increase in the case of the former but not the later uptake. However, the magnitude of the increased K+ efflux was always significantly less

than the magnitude of the AIB uptake. The Na+ efflux was then calculated from the measured influx and net flux, and was found to be increased during AIB uptake. The sum of the increase in sodium and potassium efflux was found to be equal to the saturable portion of AIB influx. These results were shown to be in accord with the ion movements that would be expected from the Na+-K+ gradient hypothesis of Crane.

3) Can other monovalent cations substitute for Na+? The results show that none of the alkali metal ions (K+, Li+, Cs+, Rb+) can produce an uptake of AIB comparable to that evidenced in the Na+ medium. However, the Li+ medium does allow a definitely concentrative uptake which is significantly greater than that evidenced in the other substitute media.

4) Can the energy in the sodium gradient drive the active transport of amino acid? In several experiments the sodium gradient was reversed, and a simultaneous concentrative uptake of AIB was evidenced. This finding eliminated the possibility that the sodium gradient is the sole source of energy in this system.

5) Can the energy in both the sodium and potassium gradients drive amino acid active transport? I have conducted experiments to measure, by means of steady-state fluxes and distribution ratios, the maximal rate of expenditure of free energy from the K+ gradient and the minimal rate of expenditure of free energy necessary for the maintenance of the amino acid gradient while the sodium gradient is simultaneously reversed. The results show that the concentrative uptake cannot be accounted for by the coupling of both ion gradients to the amino acid transport.

It appears that, although sodium and potassium do combine with and move with the carrier, the ion gradients do not provide all the energy necessary to account for the active transport process. Therefore, it becomes necessary to assume that there is also some other coupling of metabolic energy to amino acid transport in the Ehrlich ascites cell.

METHODS

A. Collection and Preparation of the Cell Suspension.

The Ehrlich ascites tumor used in these experiments is a hypotetraploid line, which has been carried in this laboratory for over 300 generations by the weekly intraperitoneal inoculation of 0.1 ml of a 2:1 dilution (with sterile saline) of the ascites into female Swiss albino mice (Millerton Farms). The usual procedure was to allow the tumor to develop for one week before killing the mice. Mice carrying the tumor for this length of time showed no signs of discomfort or tenderness although their abdomens were distended. Also the coats of these mice had a normal healthy appearance. On the day of each experiment 25 mice, inocculated 7 days previously, were killed by cervical dislocation, and their abdominal cavities were opened. At this point mice bearing excessively bloody tumors were discarded. The ascitic fluid was removed from the remaining mice by pipette (50-100 ml total volume) and was put into 30 ml of one of the buffer solutions described below, to which had been added 0.2 mg heparin chloride. At this point further preparative procedures were conducted by two different routes: the room temperature procedure or the cold shock preparation.

Room temperature preparation.-- In this procedure no osmotic shock was used, and all operations were carried out at room temperature (20-25 C). The collected ascites was diluted by the addition of an equal volume of one of the buffer solutions, and the resulting suspension (160-250 ml) was passed through pre-washed cheese cloth. The suspension was then placed in four or six 50 ml centrifuge tubes and centrifuged in an International Model K Centrifuge at 65 x g for 10 min. The supernatant and the top layer of cells in each centrifuge tube were then removed by means of a suction pipette and were discarded. The cells were resuspended in the appropriate buffer solution and again centrifuged at 65 x g for 10 min. This washing procedure was carried out twice. After the final centrifugation, the supernatant and top cell layer of each centrifuge

tube were removed by suction pipette, and all the cells remaining were resuspended in the appropriate buffer solution to an estimated cytocrit of 0.24 to 0.30, which provided for a final cytocrit of 0.09 to 0.14 when this suspension was diluted with the test medium as described later.

Cold Shock Preparation.-- In those experiments in which it was desired to raise intracellular sodium and deplete intracellular potassium, the ascites were chilled in an ice bath during the collection, and all subsequent procedures were conducted in the cold room (0-5 C). The suspension was diluted by the addition of an equal volume of chilled, demineralized water (osmotic shock) and was then run through pre-washed cheese cloth and allowed to stand in the cold for 10 min. Next the suspension was centrifuged in the cold at 65 x g for 10 min, and the supernatant fluid was discarded. The cells were then resuspended in the appropriate buffer to which ouabain had been added (as described below) to a final concentration of 2.5×10^{-4}M. The cells were then centrifuged at 65 x g for 10 min. The same wash procedure was repeated once, and the cells were then resuspended to a cytocrit of 0.24 to 0.30 in the ouabain buffer.

B. Composition of Buffered Solutions.

Several modified versions of a basic Krebs-Ringer phosphate buffer solution (hereafter, KRP) have been used. The solution routinely used was made up by adding 10 ml of a phosphate buffer solution (pH 7.0) to each 46.5 ml of a basic salt solution. The basic salt solution was comprised of: 145 mM NaCl, 5.76 mM KCl, 1.03 mM $CaCl_2$, 1.44 mM $MgSO_4$ and 1.44 mM KH_2PO_4. However, in several experiments the 145 mM NaCl was replaced by an osmotically equivalent concentration of KCl, CsCl, RbCl, LiCl or Choline chloride. The resulting buffer solutions have been abbreviated respectively: K-KRP, Cs-KRP, Rb-KRP, Li-KRP and Ch-KRP. In most cases, the phosphate buffer added to the basic salt solution was made up from stock solutions of 154 mM KH_2PO_4 and 100 mM K_2HPO_4, and the resulting buffer is noted simply

as KRP, e.g. Li-KRP. However, in some experiments one or more of the buffers were made up using the corresponding sodium phosphates; these are noted by (Na+ phosphates) following the KRP, e.g., Na-KRP (Na+ phosphates). One experiment was conducted using a bicarbonate buffer. This buffer was comprised of the same salt solution described above but with part of the NaCl osmotically replaced by $NaHCO_3$ to give a final bicarbonate concentration of 9.5 mM. When gassed with 5% CO_2 at 745 mm Hg this buffer, which will be referred to as Na-KRB, had a measured pH of 7.0. The pH of all buffers was measured in each experiment using the Beckman Expanded Scale pH Meter, and the average for all buffers in all experiments was 6.99 ± 0.02 (S.D.). Any buffer solution with a pH outside the range 6.95-7.05 was adjusted to 7.00 with the appropriate phosphate solution. Furthermore, the osmolarity of all buffer solutions was measured using a Fiske Osmometer (freezing point depression method), and the average was 286.5 ± 3.4 (S.D.) mOsmolar. Determinations of buffer sodium and potassium were done after each experiment as described below. The average sodium concentration in Na-KRP made up with sodium phosphates was 143 mM; in Na-KRP made up with potassium phosphates it was 111 mM; and for KRP solutions in which sodium was replaced by another cation, 0 mM. Average potassium concentrations in KRP solutions using sodium phosphates and in those using potassium phosphates were 7.1 mM and 33.2 mM respectively.

To make up buffer solutions containing ouabain at 2.5×10^{-4}M, 127.6 mg ouabain were dissolved in 7 ml of absolute ethanol. One ml of this solution was then added for each 100 ml of buffer solution. Ion, pH and osmolarity checks were done on the buffer after this addition.

C. Biochemicals and Labeled Compounds.

Unlabeled alpha-aminoisobutyric acid (henceforth, AIB), L-phenylalanine and ouabain were obtained from Calbiochem as A grade biochemicals.

The ^{22}Na was obtained from Nuclear Chicago Corporation in two batches, each of 0.2 mc in approximately 1.5 ml of aqueous NaCl solution. Specific activity was 60 mc/mg Na. This stock solution was diluted to 20 μc/ml by the addition of isotonic NaCl solution, and 0.2 to 1.0 of this dilution was used per experiment (approximately 90 ml of cell suspension).

Tritiated AIB (generally labeled) was obtained from Nuclear Chicago Corporation in one batch (No. 3) of 5 mc in 5 ml aqueous solution with a specific activity of 131 mc/mmole. This stock solution was diluted with isotonic NaCl solution to 100 μc/ml, and 0.2 to 1.0 ml of this dilution was used per experiment.

L-Phenylalanine-^{3}H (generally labeled) was obtained from Nuclear Chicago Corporation (Batch No. 3) as 0.5 mc (specific activity 750 mc/mmole) in aqueous solution. This stock solution was diluted to 100 μc/ml with isotonic NaCl solution, and the same amounts were used per experiment as ^{3}H-AIB.

The ^{42}K was obtained on the day of the experiment as ^{42}KCl from Phoenix Memorial Laboratories (University of Michigan) in lots of 0.5 - 1.0 mc in 1 -2 ml of isotonic KCl solution with a specific activity of greater than 56 mc/mmole KCl. The entire batch of ^{42}K was diluted with 50 ml of Na-KRP. Then, since the preparation of the ^{42}KCL at Phoenix Laboratories involved a process of dissolving in HCl and neutralizing, the pH of the buffer was checked and adjusted to 7.0 if necessary. This 50 ml of buffer was then used to suspend 20 - 25 ml of packed cells as described below.

D. Experimental Detail.

Sodium influx experiments.-- In those experiments in which the entry of ^{22}Na and ^{3}H-AIB or ^{3}H-L-phenylalanine were measured simultaneously, the following procedure was used. Three ml of the cell suspension, prepared to a cytocrit of 0.24 to 0.30 as described above, was placed in Arm A of each of several two-armed Heinecke tubes. In Arm

B was placed 4 ml of the test solution. This test solution was comprised of a combination of three stock solutions: (1) The appropriate KRP or KRB solution; (2) a corresponding ^{22}Na labeled buffer solution, and (3) a solution of amino acid in a concentration 3.5 times that desired (mixing gave a 2:7 dilution of the amino acid solution). The combination of stock solutions making up the 4 ml added to Arm B varied within each experiment as follows: for background, 4 ml of pure KRP or KRB solution was used; for ^{22}Na controls, 2 ml of ^{22}Na solution and 2 ml of KRP; for amino acid controls (necessary for double label counting procedures), 2 ml of the amino acid solution and 2 ml of the KRP; and for the double-label samples, 2 ml of the amino acid solution and 2 ml of the ^{22}Na solution were added. In experiments in which four concentrations of the amino acid were used, the stock solution of the amino acid was prepared with labeled and non-labeled amino acid to a concentration of 87.5 mM, and this stock was diluted with the appropriate KRP so that the final concentrations after mixing in the Heinecke tubes (2:7 dilution) would be 25, 10, 5, and 1 mM.

Experiments in which ^{42}K influxes were measured were conducted in the same manner as described above except that a stock KRP with ^{42}K added was used in place of the ^{22}Na-KRP. The Heinecke tubes were all placed on a rack; this allowed simultaneous mixing of the suspension in Arm A with the solution in Arm B by rotating the rack. The tubes were preincubated in a water bath at 37 C (all experiments except one were conducted at this temperature) for 2 min, the contents were then mixed and incubated for 1 or 2 min. At the end of the incubation period the tubes were immediately chilled in an ice bath for thirty seconds (thermocouple measurements showed that the temperature of the suspension in the Heinecke tubes reached 15 C within 10-11 sec). The tubes were then transferred to a cold room (0-5 C), and the following samples of each suspension were taken by means of Ostwald-Folin pipettes: 2 ml were pipetted into 75 x 10 mm tared test tubes for wet and dry weight determination; 2 ml into 100 x 13 mm test tubes for

sodium and potassium determination; 1 ml for pellet analysis was pipetted into a 125 x 15 mm test tube containing 12 ml of cold Ch-KRP. Also cytocrits of each suspension were taken by transferring an appropriate volume to Wintrobe hematocrit tubes by means of a syringe and long needle. All these samples were immediately centrifuged for 20 min in a Precision Vari-Hi-Speed Centricone at 1880x g. The supernatants of the tared test tubes and the 100 x 13 test tubes were saved for analysis of extracellular label and ion concentrations respectively; other supernatants were discarded. Next the tubes were drained for 20 min in the cold, and were then wiped with gauze to within 5 mm of the pellet. The tared tubes were weighed to determine pellet wet weight, and after 36 hr in an oven at 120 C were weighed again to determine pellet dry weight. The pellet for sodium and potassium determination was handled in the manner described later. One ml of the tared tube supernatant was extracted with 4 ml of 95% ethanol containing 0.025% (w/v) barium acetate; the 1 ml pellet (from the 125 x 16 mm test tube) was extracted with 4.5 ml of 75% ethanol with 0.106% (w/v) barium acetate. The resulting suspensions were centrifuged in an International Model K Centrifuge for 10 min at 150 x g. The supernatants were saved and the pellets of both pellet and supernatant samples were resuspended in 4.5 ml of 75% ethanol containing 0.001% (w/v) barium acetate; these suspensions were centrifuged as before. Both the original and this wash supernatant were combined and brought to 10 ml by the addition of 75% ethanol. Next 1 ml of each solution were pipetted into liquid scintillation counting vials containing 14 ml of toluene-ethanol scintillation fluid. Two 1 ml samples of each of the original amino acid stock solutions and the ^{22}Na or ^{42}K stock solutions were handled in the same way as the supernatants to give activity standards. For ^{42}K determinations in the deep-well gamma counter, 2 ml of the ethanol solution were pipetted into 125 x 15 ml test tubes which were then covered with Parafilm. Cell counts of the original Heinecke tube suspensions were also obtained using blood dilution pipettes and a hemocytometer.

^{42}K Efflux Experiments.-- The same basic procedure was used in these experiments as in the ^{22}Na influx experiments. However, after the last centrifugation of the suspension in the preparative procedure, the ascites were resuspended in 50 ml of Na-KRP (Na+-phosphates) containing the ^{42}K prepared as described above. This suspension was allowed to stand at room temperature (20-25 C) for 1 hr and was then centrifuged and washed twice with Na-KRP (Na+-phosphates) as described above, in order to remove as much extracellular label as possible. This suspension of cells preloaded with ^{42}K was then put in Arm A of the Heinecke tubes as described previously. No control containing only the labeled amino acid was necessary since simultaneous double label counting was not done (see Radiation Counting Procedures).

In triple-label experiments the cells were preloaded with ^{42}K and then placed in Arm A of the Heinecke tubes as described. However, in these experiments Arm B contained either ^{22}Na solution of the tritiated AIB solution or both as described for sodium influx experiments.

Time studies.-- In several experiments the uptake of the amino acid alone was determined for several different incubation periods. The preparation of the cells was standard according to the room temperature or cold-shock procedure. As before, 3 ml of the cell suspension (cytocrit of 0.24 to 0.30) was used per sample. However, instead of Heinecke tubes, flasks with side arms were used. These flasks allowed the suspension to have a greater surface area in contact with the air; this provided for an increased oxygen diffusion into the suspension needed for the longer incubation periods. The cell suspension was placed in the bottom of the flasks. In the arm of the flask were placed either 4 ml of KRP (background) or 4 ml of the amino acid stock solution with tritium label. The amino acid KRP for these experiments was made to a concentration of 1.75 times the desired final concentration (4:7 dilution resulted from mixing). The flasks were preincubated for 2 min in a water bath at 37 C, then mixed and incubated for varying lengths of time as described in RESULTS. At the end of their respective

incubation periods, tubes were pulled off the rack, chilled and processed as described for sodium influx experiments.

Other time studies were done in the presence of a reversed sodium gradient to determine amino acid uptake. In these experiments the intracellular sodium was raised and the intracellular potassium lowered by cold shock preparation. Then the washing procedures and the final suspension were carried out using K-KRP or Ch-KRP. The rest of the time-study proceeded as described above except that the amino acid solution was made up using the K-KRP or Ch-KRP.

E. Radiation Counting Procedures.

The analysis of tritium and ^{22}Na was carried out with the use of a Nuclear Chicago Model No. 725 liquid scintillation system. All samples were counted at least long enough to obtain a standard error of the counting rate of less than 1% in all channels used. Those samples containing only the tritiated amino acid were counted using a channels ratio procedure and quenched standards of known disintegrations per minute (dpm) supplied by Nuclear Chicago Corporation. The efficiency of tritium counting by this method was in the range 8.7 - 13.6% for all experiments, and the maximum range in any one experiment was 2.1%; however, the maximum efficiency difference between any two supernatant or any two pellet samples in any one experiment was less than 0.5%.

Since no quench standards for ^{22}Na were commercially available, it was necessary to prepare them from a standardized $^{22}NaCl$ solution of known dpm (New England Nuclear Corporation). Equal amounts of the standard solution were carefully pipetted into each of five scintillation vials by taring and reweighing the vials. Then 1.5 ml of different mixtures of ethanol and water were added to the vials. The ratio of ethanol to water in these mixtures varied from 1.4:0.1 (minimum quenching) to 1.1:0.4 (maximum quenching). Then 13.5 ml of toluene-ethanol scintillation fluid were added, and the vials were sealed by annealing.

Next the liquid scintillation counter was set for optimal counting of ^{22}Na by channels ratio counting procedure as described in the Nuclear Chicago Liquid Scintillation Manual. ^{22}Na emits a positron of approximately the same energy spectrum as the beta emission of ^{14}C, and the settings determined for ^{22}Na were found to be close to those used for ^{14}C. The ^{22}Na quench standards were counted, and the channels ratio obtained was found to be a linear function of the efficiency of counting, which ranged from 50 - 67% in the quenched standards. The samples from experiments had efficiencies in the range of 59.2 - 62.0% for all experiments. The maximum efficiency range in any one experiment was 2.0%; however, as for tritium counting, no two supernatants nor pellets in any one experiment differed by more than 1.0% in counting efficiency.

The theory for the differential determination of ^{14}C and ^{3}H activity in samples containing both labels has been discussed by Okita et al. (1957), and the same general procedure was used for the differential determination of ^{22}Na and ^{3}H in these experiments. This was warranted by the similarity of the ^{22}Na and ^{14}C spectra, and since ^{22}Na has a higher average energy of emission than ^{14}C, an even better separation from the ^{3}H spectrum was possible. Basically, this procedure involved setting up two counting windows in the counter, one encompassing the peak of the ^{14}C energy spectrum (Channel 1), the other encompassing the peak of the ^{3}H energy spectrum (Channel 2). The singly-labeled samples in each experiment (the activity of which had been previously determined by the above channels ratio procedures) were used to preset the counter so that the ^{22}Na efficiency in Channel 2 was less than 3.0%, and the ^{3}H efficiency in Channel 1 was less than 0.3%. Then all experimental samples were counted in both channels simultaneously. Since the dpm for each singly-labeled sample was already known, the ^{22}Na and ^{3}H efficiency in both channels could be calculated. Within each experiment these four efficiencies so determined for all pellet samples varied less than 2% (of the efficiency percentage), and this held true for the supernatant and standard samples. Over all experiments: the ^{22}Na efficiency in Channel 1 was 54.9 -

59.2%; in Channel 2, 0.0 - 5.3%; the ^{3}H efficiency in Channel 1 was 0.0 - 3.0%; in Channel 2, 8.7 - 13.6%. For doubly-labeled samples the counting rate in each channel is given by a linear equation in the dpm from ^{22}Na and ^{3}H: $C_i = E_{iA}d_A + E_{iB}d_B$, where E_{iA} and E_{iB} are the efficiencies of counting isotope A and B respectively in Channel 1, and d_A and d_B are the dpm for isotopes A and B. The average efficiencies obtained for the singly-labeled samples were used to solve for the dpm due ^{22}Na and the dpm due to ^{3}H by means of the two simultaneous linear equations. This method is very accurate as evidenced by the percentage of recovery of labels reported below.

A Nuclear Chicago Model 1810 Radiation Analyzer (deep-well gamma counter) was used to count ^{42}K activity. Since ^{42}K had a short half-life (12.4 hr), the time elapsed from the beginning of the counting process was recorded after each sample had been counted. In the data processing program the count-rate for each sample was converted back to a "0-time" count-rate. In experiments in which only ^{3}H and ^{42}K were used these count rates were used directly without further modification. However, since ^{22}Na emits a gamma ray in addition to a positron, in triple-label experiments the samples were counted once on the day of the experiment and again one week later. After one week the ^{42}K had decayed to less than 0.01% of its original count-rate, whereas the ^{22}Na count-rate was not significantly altered; therefore, the difference in the two count-rates was the ^{42}K count-rate. The differences were corrected for decay during counting time by applying the time elapsed for each sample on the first counting day. As a precaution all liquid scintillation samples containing ^{42}K were allowed to decay for one week before counting since strong gamma radiation does affect the liquid scintillation detection system. Levels of ^{22}Na higher than used in any experiment (but still lower than ^{42}K levels used) were also tested but did not affect background or 3H count rates in the liquid scintillation system.

F. Calculation of Results.

From the dpm obtained for ^{22}Na, ^{3}H and ^{42}K, and from the dpm of the activity standards, it was possible to determine the total amount of the given substance that had entered or left the cell. A predetermined curve relating pellet inulin space to pellet wet weight allowed an estimate of the pellet extracellular space to be made. Total pellet water was determined as the difference between the pellet wet and dry weights. When these quantities were calculated, the intracellular concentration of a substance could be determined by the following relationship (Jacquez, 1961):

$$C_i = \frac{B - C_{pe} W_e}{W_t - W_e} \qquad (1)$$

where:

B = total mmoles of substance in pellet

C_i = intracellular concentration of the substance

C_{pe} = concentration of the substance in the pellet extracellular volume

W_t = total pellet water

W_e = pellet extracellular water

Note that since the pellets were prepared by dilution of one ml of incubated ascites suspension in 12 ml of KRP solution, the pellet extracellular concentration of the substance in question (C_{pe}) is equal to one-thirteenth of the original extracellular concentration (C_e). Thus, the correction for pellet extracellular contamination ($C_{pe}W_e$) becomes much less critical.

Extracellular amino acid was always determined in mmoles/liter solution, whereas intracellular concentrations were always computed as mmoles/ kg H_2O. However, at the concentrations used in these experiments these two expressions of concentration are not significantly different. For expediency, intracellular concentrations have often been

expressed as mmoles/liter for amino acids and meq/liter for ions.

In many experiments it was desirable to calculate the influx or efflux rate of the substance in question. Fluxes, both the influx of extracellular label and the efflux of preloaded intracellular label, were calculated from the amount of activity appearing on the "cold" side during the incubation and the specific activity of the particular isotope on the "hot" side. Conventionally, fluxes are given in units of mmoles/unit area per min. I calculated fluxes in this manner in each experiment by obtaining an estimate of the cell surface area from the cell count and cytocrit measurements. These calculations assumed the cells to be spherical; however, the Ehrlich cell membrane is actually highly convoluted and irregular, and the surface area calculated must be significantly underestimated. Also the cell counts were found to be much less reproducible than measurements of the dry weight. Because this limits the accuracy of determining conventional fluxes, I decided to express my results as μmoles/g dry wt-min. This sacrifices some of the intuitive grasp of the flux parameter but it also increases the reproducibility of the measurement. Technically this expression of uptake or loss rate is not a flux; however, in the ensuing text, in order to indicate that the measured uptake or loss is an approximation to the one-way movement of a substance, I have used the terms influx and efflux in association with the units μmoles/g dry wt-min. A table of conversion factors has been provided (Appendix, Table 17), which gives the coefficients for converting the units μmoles/g dry wt-min to the other commonly used "flux" units: μmoles/cm^2-min, μmoles/kg H_2O-min and μmoles/10^7 cells-hr.

As a check on experimental accuracy percentages of recovery for labeled material were calculated for every sample in every experiment. As expected this percentage showed significantly less variation within any one experiment than over all experiments. The mean percentages of recovery were as follows: 98.53 $\pm$ 0.154 (S.E.M.) for singly-labeled ^3H samples which was not significantly different from the mean of 99.11 $\pm$ 1.12 (S.E.M.) for doubly-labeled ^3H samples; 98.80 $\pm$ 1.05 (S.E.M.) for

^{22}Na singly-labeled samples which was not significantly different from 99.23 ± 0.30 (S.E.M.) for doubly-labeled samples. Percentages of recovery estimates could not be easily made for preloading experiments which comprised the major part of the ^{42}K usage. However, in the three experiments in which ^{42}K was measured as an influx, the percentage of recovery was 98.33 ± 0.14 (S.E.M.).

In each experiment, each Heinecke tube had at least one replicate and often two. Therefore, most values given in the following tables and graphs are means of two or three replicates, and to these are appended the standard deviation of the sample set. Whenever a standard error of the mean is appended, the notation (S.E.M.) will be used, whereas no designation of the standard deviation will be made. For all ^{3}H-labeled samples the coefficient of variation (standard deviation of the mean divided by the mean) averaged 2.73 for duplicate samples and 2.51 for triplicates; for all ^{22}Na samples the coefficient of variation was 2.25 for duplicates and 2.97 for triplicates. A greater coefficient of variation for the triplicates might be expected since triplicates were usually employed in situations where error was expected to be higher. The coefficient of variation for ^{42}K samples, all of which were duplicates, was 3.05. 'Student' t-tests were routinely done for all pairs of means within each experiment; significance was not attached to the difference between any two means unless the difference by the t-test was significant at least with $P < 0.05$.

G. Sodium and Potassium Determinations.

Samples for sodium and potassium analysis were obtained from each Heinecke tube as described above (sodium influx experiments). After centrifugation this sample consisted of a supernatant fraction for the analysis of extracellular ion concentrations, and a pellet fraction for the determination of intracellular ion concentrations. 0.5 ml of the supernatant was pipetted into a 50 ml volumetric flask, and was brought up to 50 ml by the addition of an aequous solution of 14.41 meq/liter

Li_2SO_4. The preparation of buffer solution samples was conducted in the same manner.

Pellets were prepared for analysis by osmotic lysing of the cells by resuspending the pellet in 4 to 5 ml of demineralized water. After 1 hour the suspension was decanted into a 25 ml volumetric flask. The test tube was then washed with another 4 to 5 ml of demineralized water and this was added to the 25 ml flask. Then 0.25 ml of an aequous solution of 1441 meq/liter Li_2SO_4 was added to each flask and the volume was brought to 25 ml with demineralized water. Several standard aequous solutions containing known concentrations of sodium and potassium and 14.41 meq/liter Li_2SO_4 were prepared. All standards and experimental samples were read using an NIL Flame Photometer with an internal lithium standard.

Supernatant and buffer sodium and potassium concentration could be obtained directly by comparison with standard readings and correction for dilution. However, intracellular concentrations had to be calculated with consideration being given to the intracellular water in the pellet and the included extracellular solution. This was done by the use of equation (1) in most instances. In the first experiments, I attempted to eliminate the contribution of extracellular medium in the pellet by washing the pellet two times with isotonic choline in the cold. However, the values of intracellular ion concentrations so obtained did not differ significantly from values obtained with the use of equation (1) without the choline wash procedure. Therefore, in most instances the later procedure was employed. This was further justified in that in most experiments the relative change in intracellular ions was of importance rather than the absolute value of the concentration. In those experiments in which the absolute intracellular concentration of Na+ or K+ was essential (as in the reversed ion gradient studies), the extracellular sodium and potassium were low thus reducing the size of the correction factor ($C_{pe}W_e$) in equation (1).

In addition, experiments were conducted to test the possible

interference of other ions in the flame photometer measurements. It was found that only the presence of lithium (in Li-KRP) interfered with the determination of Na^+ and K^+. Therefore, no K^+ or Na^+ measurements were made for experiments involving Li-KRP.

The same samples prepared for reading on the flame photometer were also used for chloride determination in some of the experiments. Chloride concentrations were measured using an Aminco-Cotlove Chloride Titrator. The readings so obtained were converted to concentrations by comparison to readings from known concentrations of NaCl and correction for dilution.

RESULTS

Part I. Change in Na+ Influx and Efflux with Amino Acid Uptake

A. The Stoichiometry of Increased Na+ Influx to Amino Acid Influx.

In order to compare the rate of Na+ entry obtained by my methods with those obtained by other investigators, I measured in two experiments the intracellular specific activity of ^{22}Na after various lengths of incubation in Na-KRP (Na+-phosphates) containing this label. The resulting data were plotted according to the method of Sheppard and Martin (1950) for a two-compartment, first-order system. The rate constants of sodium entry subsequently calculated were: 5.0-5.5/hr for cells prepared by the room temperature procedure and 4.0-4.5/hr for cells prepared by the cold shock procedure. Maizels et al. (1958a) previously calculated the rate of sodium influx in the Ehrlich ascites cell by purely chemical means, and they reported a rate constant of 2-4/hr. More recently, Aull and Hempling (1963) also employed ^{22}Na to measure the influx rate constant. Their more thorough analysis revealed two components of sodium entry, both first-order processes: a small fast component with a rate constant of 19.4/hr, and a larger more slowly equilibrating component with a rate constant of 3.71/hr. Since the methods used in these experiments could not accomodate extremely short incubation periods, it is primarily this slower component that is shown to change with amino acid uptake; however, according to Aull and Hempling, this represents the major intracellular compartment.

It was obvious that the rapid entry of Na+ into the Ehrlich cell would make it difficult to measure accurately the absolute influx of Na+; therefore, my first experiments were designed merely to test whether the rate of Na+ entry was increased by the simultaneous influx of an A system (sodium-dependent) amino acid. The test amino acid chosen was alpha-aminoisobutyric acid (AIB) since essentially all of the saturable

component of this amino acid's entry is sodium-dependent (Inui and Christensen, 1966), and it is not readily metabolized (Christensen and Jones, 1962). In order to maximize any increase occurring in the Na+ entry, an extracellular AIB concentration of 25 mM (approximately 10 times the K_m) was used. Table 1 shows that the 2-min influx of Na+ (column 6) from a Na+-KRP (Na+-phosphates) medium was very definitely increased by the simultaneous uptake of AIB when compared to controls in which no AIB was present. For comparison, the same experiment was carried out with L-phenylalanine as the test amino acid. L-phenylalanine has been characterized as an L system amino acid whose uptake has little sensitivity to the extracellular sodium concentration, and as seen in Table 1 (experiments 11a and 11b) the uptake of this amino acid had no significant effect on the rate of sodium entry. Examination of the swelling index (ratio of grams intracellular water to grams dry weight) of samples within any one experiment showed that those incubated with amino acid had no evidence of shrinkage or swelling of the cells when compared to controls. The experiment in which L-phenylalanine was the test amino acid provides a further control to eliminate the possibility that the increase in Na+ influx during AIB uptake might be due to some less specific effect such as a difference in the extracellular osmolarity.

The increase in Na+ influx observed with AIB uptake (column 7) was also compared to the total AIB influx, and the former was always less than the latter quantity. However, as demonstrated by Inui and Christensen (1966), the total AIB uptake is the sum of two components. One of these components increases linearly with increasing extracellular AIB concentration and may be due to simple diffusion. This component is not sensitive to the extracellular sodium concentration and will be referred to as the linear component of AIB uptake. The second component saturates at high AIB concentrations, and it can be eliminated by the removal of Na+ from the extracellular medium; this component will be referred to as the saturable component of AIB uptake. In subsequent

TABLE 1

CHANGE IN Na^+ INFLUX WITH AMINO ACID UPTAKE.[1]

(1) Exp. No.	(2) Amino Acid[2]	(3) Prep-aration[3]	(4) $[Na+]_e$	(5) $[K+]_e$	(6) Na+ Influx	(7) Change in Na+ Influx (Influx - Control Influx)	(8) Total Amino Acid Influx	(9) Saturable Amino Acid Influx[4]
			meq/liter		μmoles/g dry wt/2 min			
3a	control	RT	145	6.3	87.09 ± 6.41	--	--	--
	AIB	RT	137	6.3	111.97 ± 2.99	+24.88	57.55 ± 1.31	45.05
3b	control	CS	145	6.3	135.00 ± 2.15	--	--	--
	AIB	CS	145	6.3	152.87 ± 11.78	+17.87	54.37 ± 5.11	41.87
4a	control	RT	145	5.8	76.99 ± 3.09	--	--	--
	AIB	RT	145	5.8	122.87 ± 3.60	+45.88	61.94 ± 3.02	49.44
4b	control	CS	140	5.8	117.85 ± 1.17	--	--	--
	AIB	CS	140	5.8	133.21 ± 2.84	+15.36	50.31 ± 0.78	37.81
9	control	RT(oua)	147	8.1	99.02 ± 14.61	--	--	--
	AIB	RT(oua)	147	8.1	131.36 ± 11.73	+32.34	50.45 ± 2.36	37.95
11a	control	RT	147	7.6	81.79 ± 2.63	--	--	--
	PHE	RT	147	7.6	74.38 ± 3.28	-7.41*	40.87 ± 0.92	28.07

11b	control	CS	148	7.8	134.68 ± 5.52	--	--	--
	PHE	CS	148	7.8	132.57 ± 5.95	-2.11*	41.74 ± 6.34	28.94
14	control	CS	42	34.0	102.31 ± 4.35	--	--	--
	AIB	CS	41	34.0	134.20 ± 1.25	+31.89	51.65 ± 4.69	39.15

[1]All incubations are for 2 min at 37 C with amino acid initially present at an extracellular concentration of 25 mM. Na+ influx was approximated with ^{22}Na.

[2]Control samples had no amino acid added. AIB denotes alpha aminoisobutyric acid; PHE, L-phenylalanine.

[3]RT denotes that the room temperature preparative procedure was used; CS, the cold shock procedure. Oua is used to identify experiments in which ouabain was present in a concentration of 0.25×10^{-4} M.

[4]Total amino acid influx minus that portion of the amino acid influx which is not sodium dependent; see text.

*Difference is not statistically significant.

experiments, which involved a range of AIB concentrations with each experiment, the coefficient of the linear uptake was determined (see below). This allowed the calculation of the saturable portion of the AIB influx as the difference of the total and the linear AIB influx. Actually, the coefficient of linear uptake was calculated from 1-min uptake studies; however, as an approximation this same coefficient was applied to the 2-min uptakes of AIB reported in Table 1. The saturable AIB influxes so calculated are given in column 9, and they should be recognized as overestimates. Upon comparing columns 7 and 9 it is seen that in at least three of the experiments reported the increase in Na+ influx closely approximates the saturable portion of the AIB influx. Of particular interest is experiment No. 14 in which the cold shock procedure was used to raise the intracellular Na+ concentration to 75-78 meq/liter, and the extracellular Na+ concentration was reduced to 41-42 meq/liter by choline-KRP replacement of part of the Na-KRP medium. In spite of this reversal of the sodium gradient, the saturable influx of AIB was still associated with an almost equal increase in the Na+ influx. In experiment 9 ouabain (2.5×10^{-4} M) was added to the extracellular medium in an attempt to reduce the Na+ efflux rate. This treatment produced a better correspondence of the increased Na+ influx and the saturable AIB influx.

Of further interest in Table 1 is the absence of any large difference in AIB influxes between samples prepared by the cold shock and by the room temperature procedure. Cells prepared by the former method had intracellular sodium concentrations of 70-90 meq/liter, whereas 30-45 meq/liter was obtained for those prepared by the room temperature method. On the basis of the sodium gradient hypothesis it would have been expected that such a two-fold difference in intracellular sodium would produce a much greater disparity in the AIB uptake than is actually observed.

After noting the close correspondence of the increased Na+ influx to the saturable AIB influx, it appeared that the major factor limiting an

analysis of the stoichiometry of these parameters was the rapidity with which sodium entered the cell. In the experiments reported in Table 1 the intracellular specific activity of the ^{22}Na reached levels as high as 45-55% of the extracellular specific activity. The effect of such a degree of equilibration would be to diminish any difference in the control and test uptakes. Therefore, subsequent incubation periods were reduced to one minute, and the chilling and sampling procedures were standardized and accelerated as much as possible. A still further reduction of the incubation period would have been impractical since it was desirable to have a sufficient uptake of the AIB to produce a readily measurable change in Na+ influx. Also other experiments showed that Na+ entry was still relatively rapid during the ice bath chilling and cold room procedures. (The rate constant of Na+ influx, measured by ^{22}Na, was approximately 2.4/hr. at 0-5C.) Nevertheless, it should be remembered that no attempt was made at any point to measure the absolute influx of Na+. The parameter of importance to these experiments was the change in Na+ influx due to the simultaneous influx of AIB. Therefore, although ^{22}Na continued to enter the cells during the sampling procedure, the effect would be approximately the same in samples with and without the AIB present. To insure that this sampling error was evenly distributed between test and control samples, the two series were alternately pipetted. Secondly, the critical samples, those for pellet and sodium-potassium analysis, were pipetted first.

Using these techniques, I conducted experiments at several different sodium concentrations (Table 2). In each experiment the influx of sodium was measured in the absence of AIB (control) and in the presence of AIB at 1, 5, 10 and 25 mM. Extracellular sodium concentrations of 13-150 meq/liter were obtained by replacing an appropriate fraction of the Na-KRP medium with Ch-KRP. Both buffers used K+ phosphates since the choline phosphates were not readily available. However, in order to eliminate any possibility of bias introduced by the high K+ concentration, two experiments (Nos. 23 and 29) were conducted in

TABLE 2

COMPARISON OF THE CHANGE IN INFLUX OF Na+ TO THE SATURABLE PORTION OF AMINO ACID INFLUX.[1]

(1) Exp. No.	(2) Amino acid[2] & Concentration	(3) $[Na+]_e$	(4) $[K+]_e$	(5) Na+ Influx	(6) Change in Na+ Influx (Influx - control influx)	(7) Total Amino Acid Influx	(8) Saturable Amino Acid Influx
	mM	meq/liter		μmoles/g dry wt/min			
13	control, 0	126.0	30.1	53.06 ± 2.22	--	--	--
	AIB, 25	126.0	32.3	81.84 ± 0.50	+28.78	38.73 ± 1.94	26.23
	AIB, 10	126.0	31.6	77.06 ± 0.36	+24.00	29.42 ± 0.57	24.42
	AIB, 5	126.0	30.6	71.88 ± 0.59	+18.82	23.12 ± 0.67	20.62
	AIB, 1	126.0	30.1	59.43 ± 0.18	+6.37	10.32 ± 0.07	9.82
15	control, 0	49.6	31.8	36.50 ± 0.96	--	--	--
	AIB, 25	48.9	31.6	59.19 ± 0.81	+22.69	34.15 ± 2.03	21.65
	AIB, 10	49.8	31.3	55.88 ± 0.26	+19.38	23.50 ± 0.06	18.50
	AIB, 5	50.8	31.5	52.25 ± 0.18	+15.75	19.11 ± 0.24	16.61
	AIB, 1	51.8	30.0	42.68 ± 1.46	+6.18	8.02 ± 0.23	7.52
16	control, 0	32.3	30.2	30.20 ± 2.31	--	--	--
	AIB, 25	32.7	30.4	47.65 ± 0.65	+17.45	27.68 ± 0.33	15.18
	AIB, 10	32.3	30.1	46.23 ± 1.18	+16.03	21.19 ± 0.93	16.19
	AIB, 5	32.7	30.3	41.46 ± 0.17	+11.26	15.91 ± 0.90	13.41
	AIB, 1	32.6	30.6	35.61 ± 0.36	+5.41	6.89 ± 0.04	6.39

17	control, 0	13.3	33.0	17.90 ± 0.86	--	--	--
	AIB, 25	13.3	33.0	30.97 ± 1.97	+13.07	26.93 ± 0.70	14.43
	AIB, 10	13.3	33.0	28.84 ± 1.85	+10.94	18.06 ± 0.45	13.06
	AIB, 5	13.3	33.0	26.57 ± 1.61	+8.67	13.40 ± 0.19	10.90
	AIB, 1	13.3	33.0	21.40 ± 0.30	+3.50	4.70 ± 0.18	4.21
18	control, 0	129.0	30.6	54.70 ± 5.06	--	--	--
	PHE, 25	129.0	30.6	49.53 ± 0.83	-5.17*	24.86 ± 1.10	12.06
	PHE, 10	129.0	30.0	51.50 ± 5.89	-3.20*	16.11 ± 0.95	10.99
	PHE, 5	129.0	30.1	54.05 ± 3.83	-0.65*	11.00 ± 1.32	8.44
	PHE, 1	129.0	31.0	53.82 ± 2.11	-0.88*	5.65 ± 1.48	5.14
23#	control, 0	143.0	7.9	60.23 ± 3.82	--	--	--
	AIB, 25	143.0	7.7	76.61 ± 0.45	+16.38	24.98 ± 0.53	17.88
	AIB, 10	143.0	7.7	75.97 ± 0.04	+15.74	20.40 ± 0.09	17.56
	AIB, 5	143.0	7.6	72.77 ± 0.69	+12.54	14.96 ± 0.05	13.54
	AIB, 1	144.0	7.6	64.32 ± 0.24	+4.09	6.02 ± 0.92	5.74
29	control, 0	150.0	4.6	44.78 ± 1.62	--	--	--
	AIB, 25	145.0	6.6	70.71 ± 0.54	+25.93	38.22 ± 1.17	25.72
	AIB, 10	145.0	6.6	69.46 ± 1.12	+24.68	29.96 ± 1.67	24.96
	AIB, 5	145.0	6.6	63.89 ± 0.04	+19.11	24.38 ± 0.34	21.89
	AIB, 1	145.1	6.6	53.79 ± 0.19	+9.01	10.57 ± 0.04	10.07

[1]All incubations were for 1 min at 37 C except Exp. no. 23 which was conducted at 27 C. Na^+ influx was estimated by $^{22}Na^+$.

[2]The same symbols are used as in Table 1.

#Conducted at 27 C instead of 37 C.

*Difference is not statistically significant.

KRP and KRB solutions using sodium phosphates. Also samples in experiment 29 were incubated at 27 C as a check on the other experiments, all of which employed 37 C incubations. In order to calculate the saturable portion of the amino acid influx (Table 2, column 8) the results of each experiment in the series and of all subsequent experiments using a range of AIB concentrations were analyzed to determine the coefficient of the linear component of the AIB uptake (K_D). This was accomplished with the use of a computer program which, by an iterative process, minimized the variance of data points around a Lineweaver-Burke plot by optimizing the value of K_D selected. Over all AIB experiments K_D was 0.50 ± 0.05 (μmoles/g dry wt-min)/mM extracellular amino acid. There was found to be no significant variation among experiments with different extracellular sodium concentrations. When converted to the units of min^{-1} (see APPENDIX for conversion factor), K_D was 0.134 min^{-1} which compared favorably with the values 0.1 min^{-1} given by Oxender and Christensen (1963) and 0.14-0.15 min^{-1} given by Inui and Christensen (1966). Figure 1 illustrates the method of obtaining the saturable AIB influx as the difference of the total AIB influx and the linear (non-sodium-dependent) portion of the AIB influx, which is determined as K_D times the AIB extracellular concentration. Figure 1 further illustrates the close approximation of the change in Na+ influx to this calculated saturable AIB influx. Table 2 shows that this 1:1 relationship held for all AIB concentrations and at each Na+ concentration used (compare values in column 6 with those in column 8). Over all 1-min flux studies the average ratio of the increase in Na+ influx to the saturable portion of the AIB influx was 0.912 ± 0.020 (S.E.M.). It was expected that this ratio would not be precisely 1.0 since the change in Na+ influx was an underestimate for the following reason. During the 1-min incubation period and the subsequent handling period the intracellular ^{22}Na specific activity reached as high as 25% of the extracellular specific activity for test samples, and 15% of the extracellular specific activity for control samples. Such a degree of equilibration would be

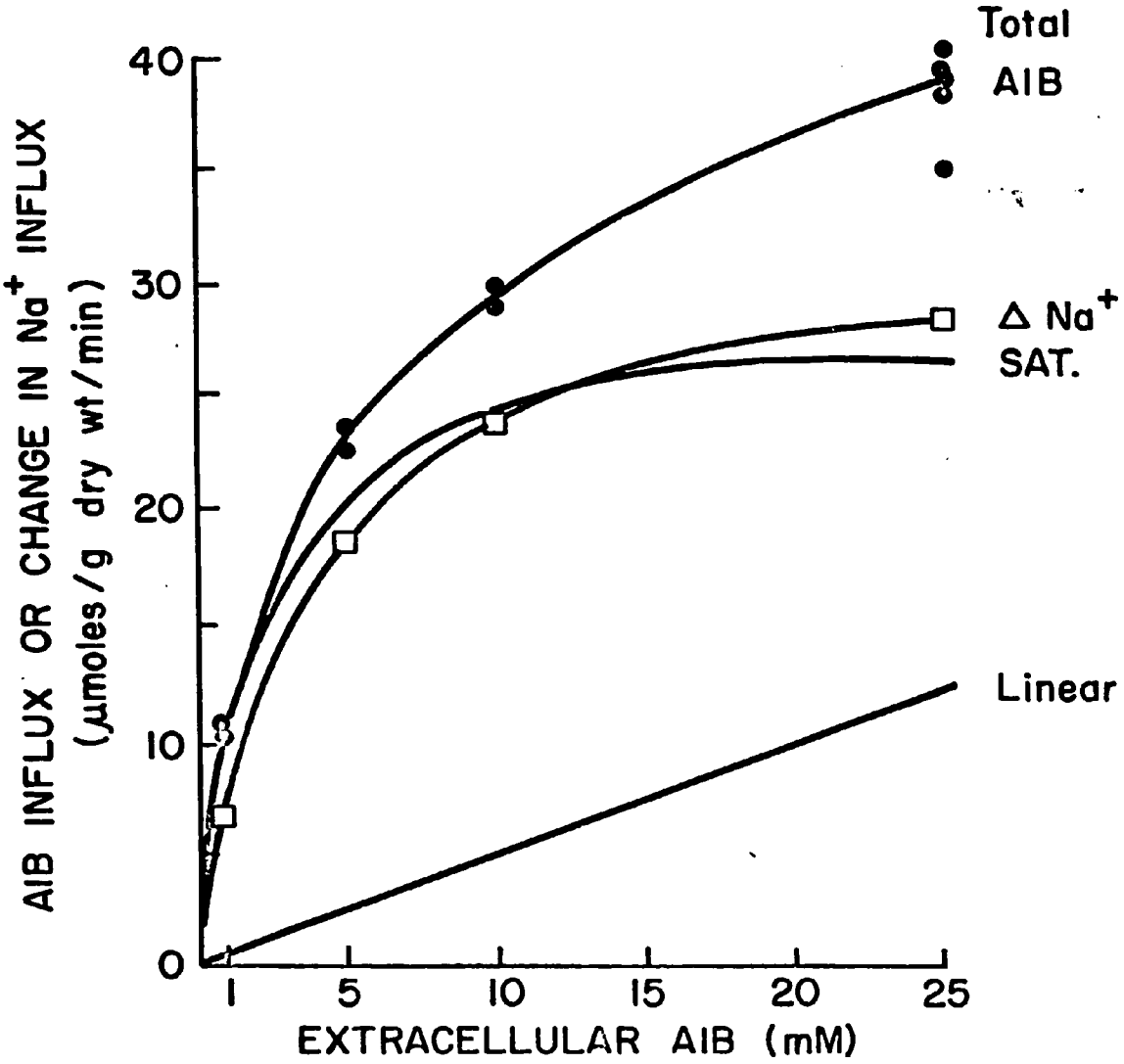


Figure 1.-- Correlation of Change in Na+ Influx with AIB Influx for 1 min incubations at 37 C. Influxes were measured for initial AIB concentrations of 1, 5, 10 and 25 mM. The Change in Na+ influx (curve designated by □ and Na+) is the difference between Na+ influx in the presence of AIB and the influx in the absence of AIB. The curve labeled SAT. is the saturable component of AIB uptake calculated by subtracting the linear component (labeled LINEAR) from the total AIB influx (labeled TOTAL AIB). Data is from Table 2, experiment No. 13.

expected to reduce the differences in Na+ influx measured. I have calculated on the basis of a simple model that a 20% equilibration of the intracellular specific activity with the extracellular specific activity would reduce the measured difference in influx to about 90% of its true value. Since in the same period there is little efflux of accumulated AIB, the measured AIB influx is a good measure of the true influx. Therefore, the calculated value of 0.912 for the ratio of the increased Na+ influx to the saturable AIB influx is compatible with a true ratio of 1.0. This 1:1 relationship is graphically illustrated in Figure 2. As the extracellular Na+ concentration decreased, the saturable (and total) AIB influx decreased, but this was matched by an equal decrease in the change in Na+ influx. Secondly, the 1:1 relationship held for both inwardly and outwardly directed Na+ gradients. Furthermore, in the experiment in which extracellular Na+ was 32 meq/liter and intracellular Na+ was in the range 41-44 meq/liter, the AIB uptake was not concentrative at the higher levels of AIB concentration, but at 1 mM AIB the intracellular concentration reached 2 mM. Thus, at this particular concentration (2 replicates) there is evidence that AIB can be concentrated in the presence of a reversed sodium gradient.

Reduction of the incubation temperature from 37 C to 27 C diminished the AIB influx (Table 2, experiment No. 23); however, the same 1:1 stoichiometry was found as it was for experiments in which Na+ phosphates were used in the buffered medium (experiment Nos. 23 and 29).[1]

One experiment (Table 2, experiment No. 18) was also conducted using L-phenylalanine as the test amino acid, and as before it produced no significant change in the Na+ influx. This provided further evidence that the increase in Na+ uptake is not a non-specific (e.g., osmotic) effect of the added amino acid. The results of these experiments show a definite 1:1 relationship of the increase in Na+ influx and the saturable

[1]The diffusion constant for AIB at 27 C was estimated as 0.284 μmoles/g dry wt-min/mM.

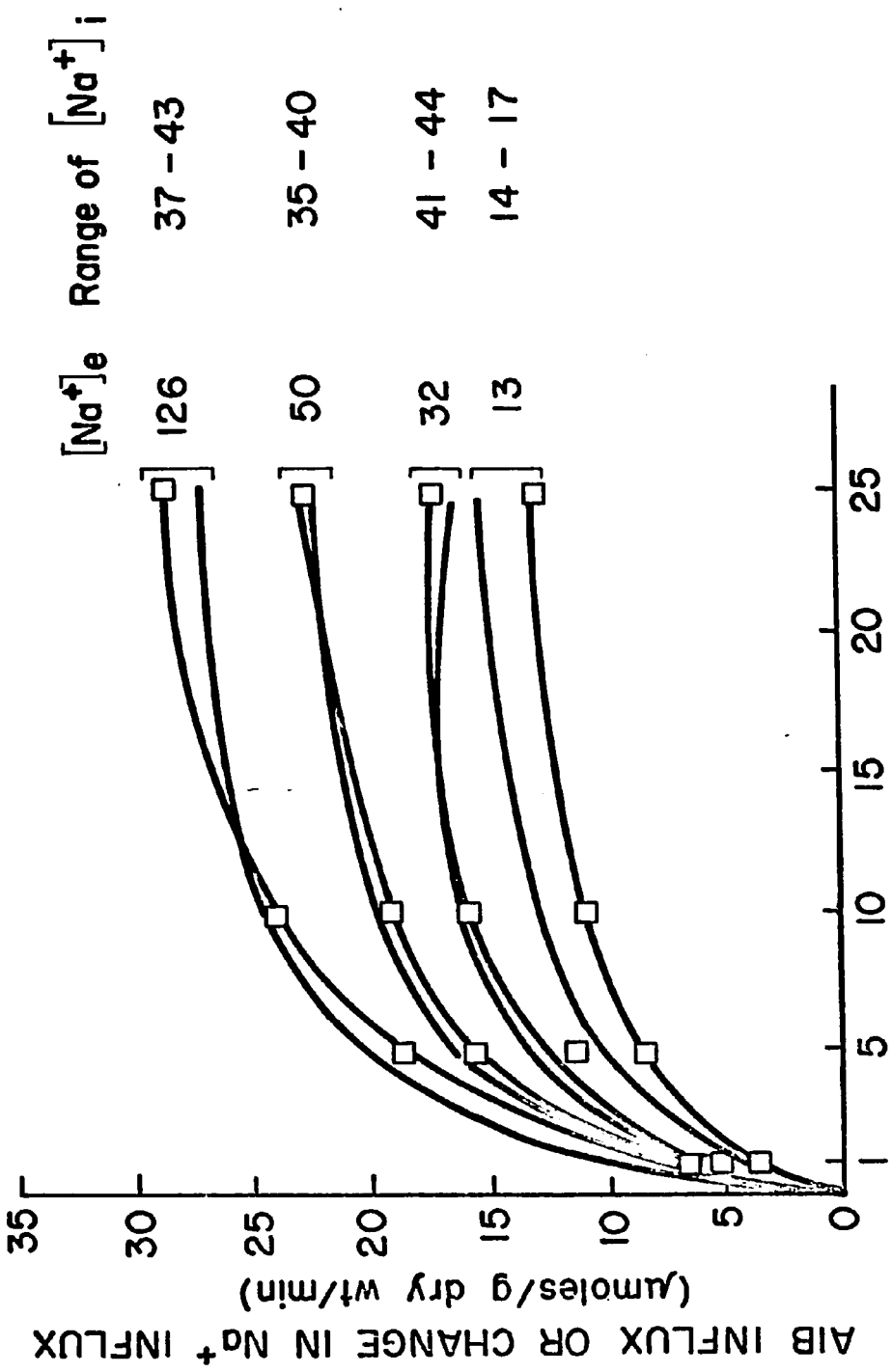


Figure 2. -- Correlation of the change in Na+ influx with the Saturable component of AIB influx at different extracellular sodium concentrations. Incubations were all for 1 min at 37 C. Influxes were measured for initial extracellular AIB concentrations of 1, 5, 10 and 25 mM. The change in Na+ influx (□ - □) and the saturable AIB influx (plot with no associated symbols) were calculated as described in Fig. 1. The extracellular and intracellular concentrations of Na+ are in meq/liter of extracellular and meq/kg intracellular water. Data are from Table 2, Experiment Nos. 13, 15, 16 and 17.

portion of the AIB influx over a wide range of Na+ and AIB concentrations, for incubation temperatures of 27 and 37 C, and for K+ extracellular concentrations of 6-33 meq/liter.

B. Correlation of Change in Na+ Efflux to Amino Acid Influx.

Upon observing a definite change in Na+ influx with AIB uptake it was desirable to then test for a possible effect of the uptake on Na+ efflux. I first attempted to measure Na+ efflux directly by the rate of exit of ^{22}Na from cells preloaded with this label. However, three experiments using variations of this method showed that, due to the tremendous permeability of the Ehrlich cell to Na+, preloaded ^{22}Na would equilibrate almost completely with the extracellular medium in the time necessary for the preparation of the incubation tubes. Considering the time involved in the preparative procedure, it was impossible to obtain meaningful data in this fashion.

The other alternative was to compute the change in Na+ efflux from the change in net Na+ movement and the change in Na+ influx reported above. This was done for all experiments reported in Table 2 using the following relationships:

$$\text{Swelling Index} = SI = \frac{\text{ml intracellular water}}{\text{g dry wt of pellet}}$$

$$\text{Net sodium movement} = \text{Net} = ([Na+]_i^t - [Na+]_i^o) \times SI$$

where: o superscript indicates a measurement before the incubation period, i.e., at "0-time."

t superscript indicates a measurement after the 1 min incubation period.

Using this convention, a positive net movement indicates a Na+ gain by the cells. From the Na+ influxes (Table 2, column 5) and the Na+ net movements the effluxes were calculated as follows:

$$\text{Net} = \text{Influx} - \text{Efflux}$$

$$\text{Efflux} = \text{Influx} - \text{Net}$$

Changes occurring during the accumulation were then computed. The subscript convention is: C = control sample (no amino acid added) and T = test sample (amino acid added).

$$\text{Change in Efflux} = (\text{Efflux}_T - \text{Efflux}_C)$$

$$= (\text{Influx}_T - \text{Net}_T) - (\text{Influx}_C - \text{Net}_C)$$

rearranging:

$$\text{Change in Efflux} = (\text{Influx}_T - \text{Influx}_C) - (\text{Net}_T - \text{Net}_C) \quad (2)$$

Thus the change in the Na+ efflux was computed as the change in the Na+ influx minus the change in the net Na+ movement. By this convention, a positive value for the change in Na+ efflux indicates an increase in Na+ efflux during the amino acid uptake. Table 3 contains the intermediate steps and the computed changes in Na+ efflux. It should be noted that the changes in net movements depended on very small differences in the intracellular sodium concentration due to the short incubation period; therefore, resolution of these concentrations became a problem and greatly limited the accuracy of the results. Nevertheless, these results (Table 3) clearly indicate: (1) There was always an increase in Na+ efflux during the uptake of AIB, but not during the uptake of L-phenylalanine. (2) The magnitude of the change in Na+ efflux tended to decrease as the change in Na+ influx decreased. (3) The increase in Na+ efflux was generally less than one-half of the increase in Na+ influx.

A better correlation of the increase in Na+ efflux to the AIB influx would have been desirable, but it appears impossible to obtain with the methods employed here. However, a pattern of an increase in Na+ efflux, which is less than the increase in Na+ influx occurring during AIB uptake, is established by these results.

C. Kinetic Analysis of Amino Acid and Na+ Influx Data.

In the experiments of Table 2, AIB uptakes were measured at

TABLE 3

CALCULATION OF CHANGE IN Na+ EFFLUX DURING THE UPTAKE OF AMINO ACID.[1]

(1) Exp. No.	(2) Amino Acid & Concentration	(3) $[Na^+]_i$		(4) Swelling Index[2]	(5) Net Na+ Movement[3]	(6) Change in Net Na+ Movement (Net - Control Net)	(7) Change in Na+ Influx[4]	(8) Change in Na+ Efflux[5] [(7) - (6)]
		"0 - time"	After 1 min					
	mM	meq/ liter			μmoles/g dry wt/min			
13	control, 0	39.4	37.2	3.80	-8.36	--	--	--
	AIB, 25	39.4	43.4	3.56	+14.24	+22.60	+28.78	+6.18
	AIB, 10	39.4	42.9	3.85	+13.48	+21.84	+24.00	+2.16
	AIB, 5	39.4	41.7	3.92	+9.01	+17.37	+18.82	+1.45
	AIB, 1	39.4	37.2	3.94	-8.62	-0.26	+6.37	+6.63
15	control, 0	42.0	35.1	3.62	-24.98	--	--	--
	AIB, 25	42.0	39.8	3.28	-7.22	+17.76	+22.69	+4.93
	AIB, 10	42.0	37.5	3.37	-15.16	+9.82	+19.38	+9.56
	AIB, 5	42.0	38.2	3.55	-13.49	+11.49	+15.75	+4.26
	AIB, 1	42.0	36.2	3.60	-20.88	+4.10	+6.18	+2.08
16	control, 0	46.6	40.9	3.36	-19.15	--	--	--
	AIB, 25	46.6	42.8	3.08	-11.70	+7.45	+17.45	+10.00
	AIB, 10	46.6	43.5	3.23	-10.01	+9.14	+16.03	+6.89
	AIB, 5	46.6	42.9	3.39	-12.54	+6.61	+11.26	+4.65
	AIB, 1	46.6	41.1	3.41	-18.76	+0.39	+5.41	+5.02

17	control, 0	35.4	14.8	3.75	-77.25	--	--	--
	AIB, 25	35.4	15.5	3.51	-69.85	+7.40	+13.07	+5.67
	AIB, 10	35.4	16.8	3.67	-68.26	+8.99	+10.94	+1.95
	AIB, 5	35.4	16.2	3.61	-69.31	+7.94	+8.67	+0.73
	AIB, 1	35.4	15.4	3.77	-75.40	+1.85	+3.50	+1.65
18	control, 0	37.6	45.6	3.71	+29.68	--	--	--
	PHE, 25	37.6	44.9	3.67	+26.79	-2.89	-5.17	-2.28
	PHE, 10	37.6	44.6	3.78	+26.46	-3.22	-3.20	-0.02
	PHE, 5	37.6	45.3	3.84	+29.72	+0.04	-0.65	-0.69
	PHE, 1	37.6	44.7	3.82	+27.12	-2.56	-0.88	+1.68
23	control, 0	43.7	56.3	3.77	+47.50	--	--	--
	AIB, 25	43.7	59.6	3.45	+54.86	+7.36	+16.39	+9.00
	AIB, 10	43.7	59.0	3.38	+51.71	+4.21	+15.74	+11.53
	AIB, 5	43.7	58.1	3.59	+51.50	+4.20	+12.54	+8.34
	AIB, 1	43.7	57.9	3.63	+51.55	+4.05	+4.09	+0.04
29	control, 0	34.1	33.3	3.84	-3.07	--	--	--
	AIB, 25	34.1	37.5	3.56	+12.10	+15.17	+25.93	+10.76
	AIB, 10	34.1	38.0	3.82	+14.90	+17.97	+24.68	+6.71
	AIB, 5	34.1	35.7	3.94	+6.30	+9.37	+19.12	+9.75
	AIB, 1	34.1	34.1	4.07	0.00	+3.07	+9.01	+5.94

[1]Data are from the same experiments as reported in Table 2. Incubations were for 1 min at 37 C, except Exp. no. 23 which was conducted at 27 C.

[2]The swelling index is grams of intracellular water divided by grams dry wt in the sample.

[3]A positive net movement indicates a net gain of sodium over the 1 min incubation period.

[4]Taken from Table 2, column 6.

[5]A positive value for this parameter indicates an increase in Na+ efflux in the test sample compared with that in the control.

different AIB concentrations and different extracellular Na+ concentrations. The influxes measured were over 1-min incubation periods, and thus they are good approximations to the initial velocity of uptake of AIB, and to a lesser extent of the change in the initial velocity of uptake of Na+. Although the experiments were not originally designed for this purpose, this data can be analyzed to determine the kinetic parameters of the uptake process. The most useful graphical method for this purpose is the Augustinsson plot as used by Wheeler et al. (1965) and Inui and Christensen (1966). As with other kinetic analyses, this method assumes that the transport process can be described by Michaelis-Menton kinetics (initial rate approximation), although this assumption is somewhat complicated by the possibility that Na+ is also a substrate for the amino acid carrier. Nevertheless, the Km constants derived from such analyses provide qualitative information as to the relative affinity of the carrier for amino acid or Na+, and the v_{max} values provide an estimate of the maximal rate of transport. The Augustinsson plot employs the following rearrangement of the basic Michaelis-Menton relationship:

$$v = v_{max} - K_m \frac{v}{S}$$

where: v = saturable portion of the amino acid initial influx or the change in Na+ influx.

S = Amino acid or sodium extracellular concentration.

v_{max} = Apparent maximal rate of transport.

K_m = Amino acid or sodium concentration giving an influx equal to one-half the value of v_{max}.

When v is plotted on the ordinate against v/S on the abscissa at different amino acid or sodium concentrations, S, the result is a straight line. The utility of this type of plot is evidenced by the fact that the slope of this line is $-K_m$ and the y-intercept is v_{max}. This permits an easy visual comparison of a family of plots. In the following graphs (Figs.

3, 4 and 5) data from Table 2 have been plotted in this manner using a computer to fit the best straight lines by simple linear regression. Figure 3 illustrates that the velocity of uptake of AIB is directly dependent on the inverse of the first power of the extracellular sodium concentration, as has been shown by Wheeler et al. (1965). In this plot the slope and y-intercept do not have an intuitively meaningful interpretation.

When this data is replotted as v_{AIB} vs. $v_{AIB}/[AIB]_e$ at different Na+ extracellular concentrations (Fig. 4), the slope and y-intercept are, respectively, the K_m and v_{max} of the carrier for AIB. It can be seen that as the intracellular Na+ falls, the v_{max} falls; however, the Na+ has much less effect on the K_m. The slopes for the various lines have been calculated and are found with the v_{max} values in Table 4.

TABLE 4

K_m and v_{max} FOR AIB OBTAINED FROM AUGUSTINSSON PLOTS

$[Na+]_e$	K_m [1]	v_{max} [2]
meq/liter	mM	mmoles/kg H_2O-min
126	2.17 ± 0.15	7.79 ± 0.21
50	2.35 ± 0.37	6.80 ± 0.44
32	1.96 ± 0.28	5.59 ± 0.29
13.3	3.14 ± 0.24	4.91 ± 0.16

[1] Calculated slopes of lines in Fig. 4.

[2] Y-intercepts from lines in Fig. 4.

Analysis of the results in Table 4 by the 'Student' t-test shows that any two of the v_{max} values are significantly different with $P<0.05$,

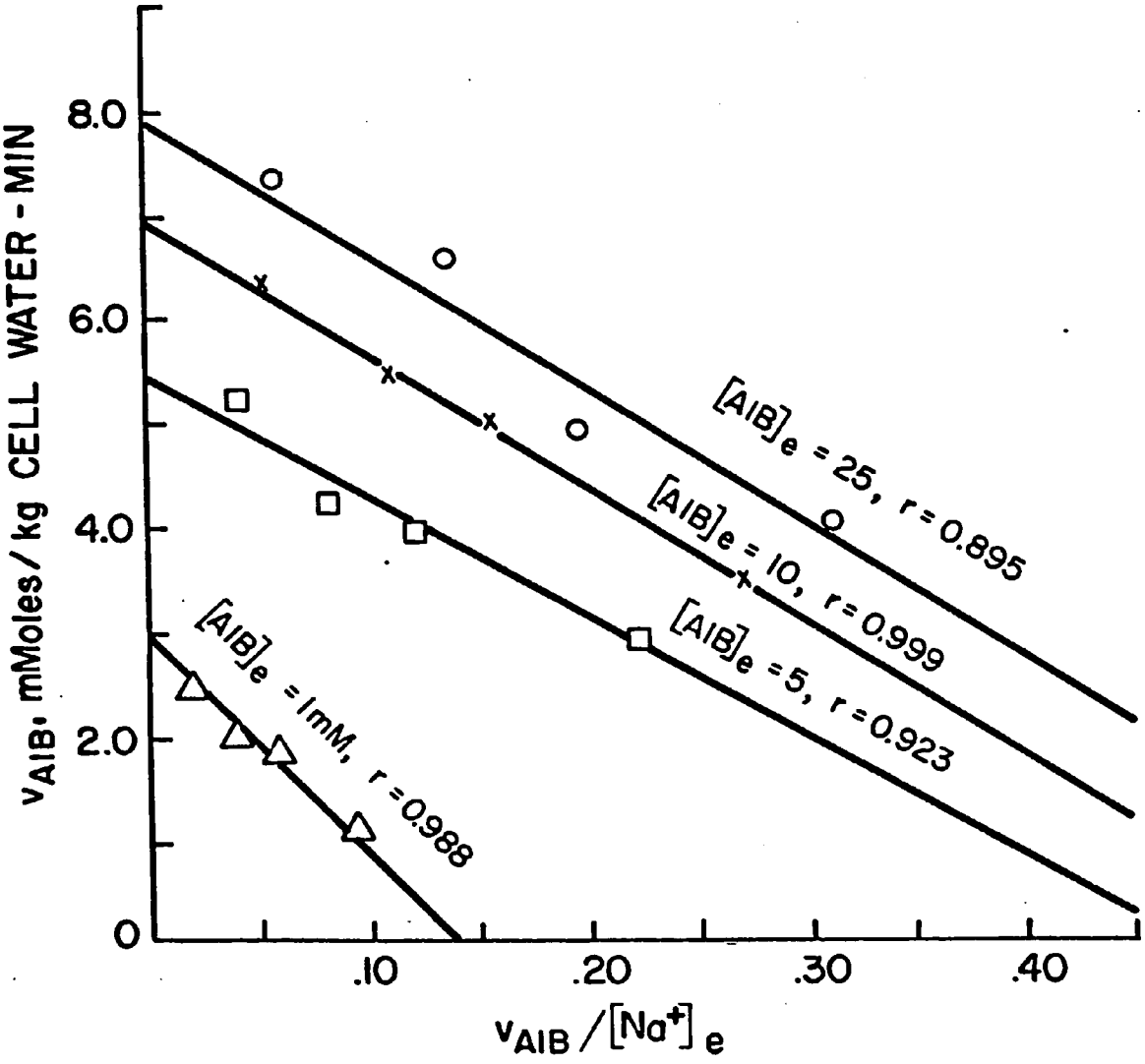


Figure 3.-- Augustinsson plots of the Na+-dependent influx of AIB at various extracellular Na+ concentrations. Influxes were measured over a 1-min incubation period at 37 C in phosphate buffer with $[K+]_e$ = 32 mM. Choline was used to substitute osmotically for Na+.

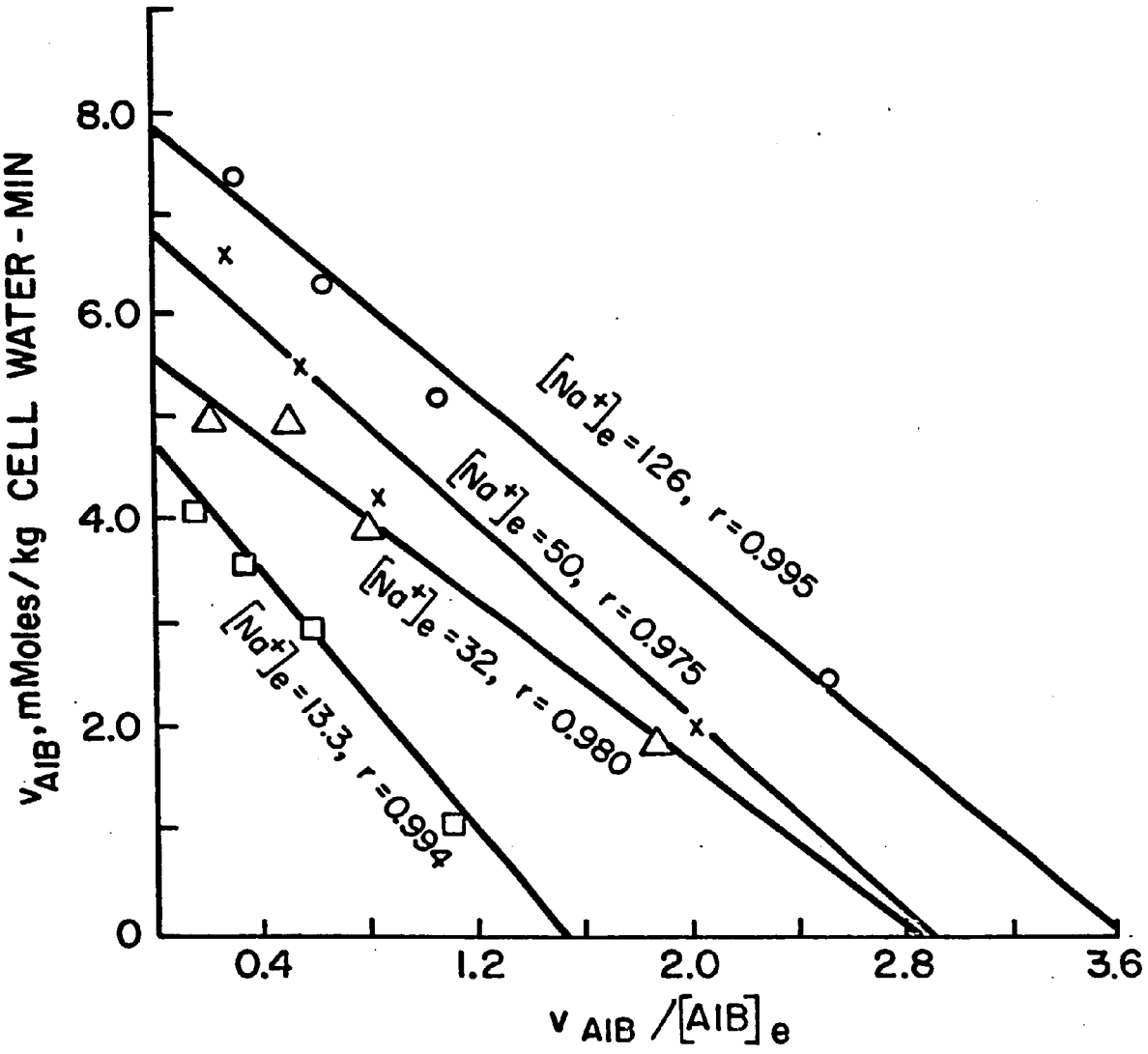


Figure 4. -- Augustinsson plots of the Na+-dependent influx of AIB at different extracellular AIB concentrations. Influxes were measured over a 1-min incubation period at 37 C in phosphate buffer with $[K^+]_e$ = 32 mM. Choline was used to substitute osmotically for Na+.

whereas only the K_m observed at the 13.3 meq/liter sodium concentration is significantly different from the other three K_m's ($P<0.05$).

If it is assumed that the increase in sodium influx observed during the uptake of AIB is due to Na+ entry on the AIB carrier, then a plot of the change in Na+ influx (Δv_{Na}) against $\Delta v_{Na}/[Na+]_e$ will provide the apparent K_m and v_{max} of this carrier for Na+. Such a plot has been made (Fig. 5), and it is seen that as the amino acid concentration decreases the v_{max} for Na+ (y-intercept) markedly decreases, whereas there is much less of an effect on the K_m (slopes). Table 5 lists the K_m's and v_{max}'s derived from Figure 5. Any two values of v_{max} in this table are significantly different at $P<0.02$, whereas no two values of K_m are significantly different.

TABLE 5

K_m and v_{max} for Na+ OBTAINED FROM AUGUSTINSSON PLOTS

$[AIB]_e$	K_m [1]	v_{max} [2]
mM	mM	mmoles/kg H_2O-min
25	20.66 ± 1.98	9.50 ± 0.36
10	18.82 ± 3.97	7.54 ± 0.59
5	17.47 ± 4.25	5.52 ± 4.85
1	11.91 ± 6.38	1.95 ± 0.29

[1] Calculated slopes of lines in Fig. 5.

[2] Y-intercepts of lines in Fig. 5.

D. Discussion: The Association of Increased Sodium Influx and Efflux with Amino Acid Influx.

From the results presented in Tables 1 and 2, and Figures 1 and 2, there is little doubt that sodium influx is increased by the simultaneous

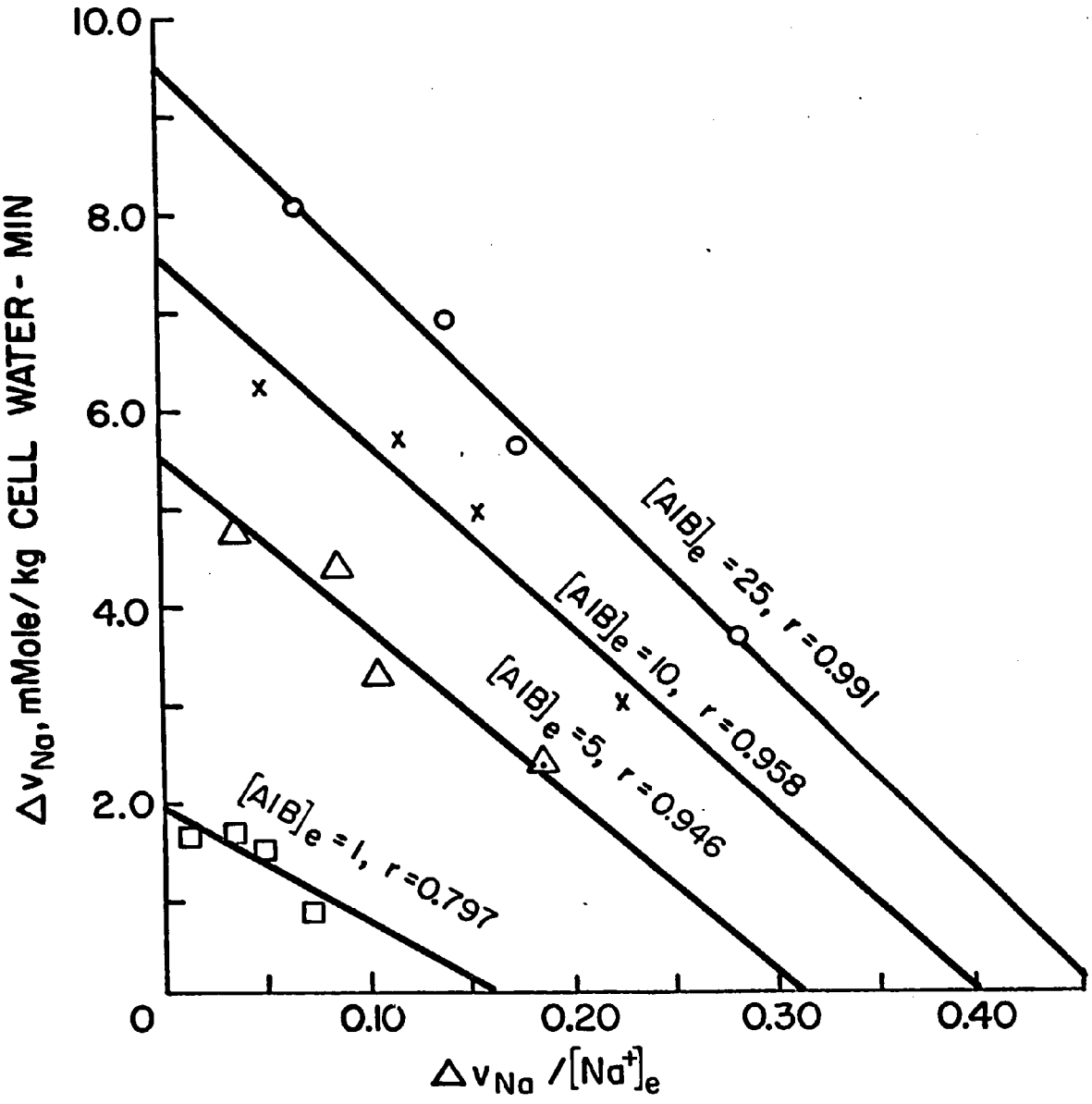


Figure 5. -- Augustinsson plots of the increase in Na+ influx during the uptake of AIB from various Na+ extracellular concentrations. All incubations were for 1 min at 37 C in phosphate buffer with $[K+]_e$ = 32 meq/liter. Extracellular Na+ is replaced to various extents by choline.

influx of AIB. Furthermore, there is ample evidence that this increase in Na+ influx is equal to the saturable (Na+-dependent) portion of the AIB influx. This 1:1 relationship holds over a wide range of sodium and amino acid concentrations, and for both inwardly and outwardly directed sodium gradients. However, the transport of L-phenylalanine, which exhibits little sensitivity to Na+, produces no change in Na+ influx. Christensen (1967) has also examined ^{22}Na influxes in the Ehrlich cell and also finds no increased Na+ influx with L-phenylalanine uptake nor with the uptake of other L system amino acids. In the avian red blood cell several investigators have reported increased Na+ influxes associated with amino acid influx of anywhere from 0.96 to 2.52 times the amino acid influx (Vidaver, 1964d; Wheeler and Christensen, 1967a).

The results in Table 3 also show there is a definite increase in Na+ efflux concomitant with AIB uptake, but not with phenylalanine uptake. However, the increase in Na+ efflux was always less than the increase in influx. Wheeler and Christensen (1967a) have also reported an increased efflux with the uptake of AIB in the pigeon red blood cell, but they find the increase in efflux is equal in magnitude to the increase in influx of Na+ so that no net Na+ movement occurs.

I interpret these results as showing that one Na+ is bound to the A-system carrier for each amino acid bound, but that no Na+ is bound to the Na+-insensitive L carrier. Presumably the A carrier in moving from the outer to the inner membrane surface would provide for both amino acid influx and an increase in Na+ influx. Furthermore, the Na+ efflux results show that at least part of the Na+ carried in is returning possibly via the carrier. According to this interpretation, sodium would be acting as a cofactor bound to some carrier site. It is unlikely that Na+ is moving independently as a counter-ion to AIB, because both AIB and phenylalanine are neutral amino acids, and one would expect Na+ to move with phenylalanine as well as with AIB. Hence if Na+ is moving as a counter-ion it must be as a counter-ion to the A carrier itself. This appears to be contradicted by the simultaneous increase in Na+ efflux

which would not be expected if Na+ were acting as a counter-ion. It seems to me a much more likely explanation of the 1:1 stoichiometry and of the Michaelis-Menton type of relationship between the AIB flux and the extracellular sodium, is that sodium and amino acid are bound to the same carrier. This has already been suggested by Schultz and Zalusky (1965) for amino acid transport in the isolated rabbit ileum, and it is in accord with the sodium gradient hypothesis of Crane (1965).

Kinetic analysis of the results has also indicated that the maximal transport velocity of the carrier (v_{max}) but not the K_m for AIB, is very sensitive to the extracellular sodium concentration. On the other hand, the v_{max} for Na+ is sensitive to the concentration of AIB, whereas the K_m of the carrier for Na+ is not altered by the AIB. I interpret this as showing that carrier movement is restricted by the absence of either amino acid or Na+ from their respective carrier sites, but that the presence or absence of either Na+ or amino acid from their sites does not influence the K_m of the carrier for the other substrate. These results are in conflict with those of Wheeler et al. (1965) and Inui and Christensen (1966) who find that when Na+ is lowered v_{max} falls but K_m also increases in the Ehrlich cell. These investigators used much lower AIB concentrations than were used in this investigation, and workers from their laboratory have previously suggested that changes in K_m for amino acids may be masked when amino acid concentrations greater than the K_m are used (Akedo and Christensen, 1962). Why this should be so is not immediately obvious to me. The Augustinsson plot, by virtue of its linearity, implies that the K_m value (i.e., the slope) will remain constant over any range of the substrate concentration.

Inui and Christensen (1966) have presented a theoretical development (using the kinetic relationships for a two substrate enzyme) of the velocity equation for a carrier with two sites, one for Na+ and one for amino acid. The equation they have derived is:

$$v = \frac{\frac{[Na+]}{A_2 + A_4\,[Na+]} \cdot [S]}{\frac{A_1 + A_3\,[Na+]}{A_2 + A_4\,[Na+]} + [S]} \qquad (3)$$

Where: A_1, A_2, A_3 and A_4 are constants.

According to this formulation, the fraction in the numerator takes the place of v_{max} in the Michaelis-Menten equation, and the fraction in the denominator takes the place of K_m. These authors have argued that equation (3) predicts a change in both v_{max} and K_m with variations in the concentration of sodium. However, a whole range of relative change in v_{max} to change in K_m might be expected depending on the values of the constants. My results show a large variation in v_{max} but not K_m with different Na+ concentrations. This would be expected from equation (3) if A_3 and A_4 are very small, or if A_1 is much greater than A_3 and A_2 is much greater than A_4, or also if A_3 [Na+] is approximately equal to A_4 [Na+] and A_1 and A_2 are comparatively small. On the other hand, if A_2 is very large and if A_3 and A_4 are significant but not equal, then large variations in K_m would be expected with changes in Na+, but with no change in v_{max}. This later result is the one found by Vidaver (1964a) for glycine uptake in the pigeon red blood cell.

Such an analysis might also account for the disparity in the results of Lyon and Crane (1966a) who found that K_m for sugar transport in the rat intestinal sac preparation increased as Na+ decreased with no change in v_{max}, and Schultz and Zalusky (1964b), who found no change in K_m but a decrease in v_{max} with a decrease in Na+ for the rabbit ileum preparation. The different results might represent a species difference (as suggested by Lyon and Crane, 1965a) which is reflected as a difference in the constants in equation (3).

Of further interest in the results presented above is the apparent lack of influence of the sodium gradient on the initial rate of AIB uptake. Large variations in the intracellular Na+ concentration seemed to have little effect on the initial rate of AIB uptake, a finding which has also been reported for the sodium-dependent uptake of amino acids by skeletal muscle (Kipnis and Parrish, 1965). This apparent contradiction to the sodium gradient hypothesis was pursued further in other experiments which will be discussed later.

The finding of increased sodium influx and efflux occurring with AIB transport made it desirable to test for any change in K+ movement which would be predicted to occur in accordance with the sodium-potassium gradient hypothesis of Crane (1965).

Part II. Changes in K+ Movements with Amino Acid Uptake.

A. Increase in K+ Efflux with Amino Acid Influx.

Crane's (1965) sodium-potassium gradient hypothesis states that potassium displaces Na+ from its carrier site when the carrier is on the intracellular side of the membrane. If the A amino acid carrier in the Ehrlich cell is operating in the same way, an increase in K+ efflux would be expected during amino acid influx. I have tested this hypothesis directly by measuring the rate of efflux of preloaded ^{42}K in the presence and absence of AIB influx. Table 6 presents the results of these experiments. The first two experiments (Nos. 25 and 27) used a room temperature preparation so as to maintain a high intracellular K+ concentration (106-124 meq/liter). In these experiments the K+ efflux was seen to increase during the uptake of AIB at least from the 5, 10 and 25 mM concentrations. This increase varied from 11-60% of the saturable portion of the AIB influx. However, in the following two experiments (Nos. 34 and 39) the intracellular potassium was decreased to 73-77 meq/liter by cold shock preparation of the cells. In these experiments there was no significant increase in the efflux of potassium during the uptake of AIB at any concentration. In the last experiment (No. 43) there was no observed increase in K+ efflux during the uptake of L-phenylalanine, although the intracellular K+ was at a 'normal' level of 120 mM. When viewed in conjunction with the results showing an increase in Na+ efflux with AIB uptake, it would appear that K+ is displacing part of the sodium ion from the amino acid carrier in the cells prepared by the room temperature procedure. Such a partial K+ displacement of the Na+ would account for the fact that the increase in Na+ efflux occurring during amino acid uptake was less than the increase in Na+ influx, and it would also account for the increase in K+ efflux. When the intracellular potassium concentration was reduced to less than 80 meq/liter there was no longer any augmentation of the K+ efflux. By continuing the above hypothesis, this would indicate that the carrier

TABLE 6

COMPARISON OF THE CHANGE IN K+ EFFLUX TO THE SATURABLE PORTION OF AMINO ACID INFLUX.[1]

(1) Exp. No.	(2) Amino Acid & Concentration	(3) $[K^+]_i$	(4) $[K^+]_e$	(5) K+ Efflux	(6) Change in K+ Efflux (Efflux - Control Efflux)	(7) Total Amino Acid Influx	(8) Saturable Amino Acid Influx
	mM	meq/liter		μmoles/g dry wt/min			
25	control, 0	106	6.6	61.30 ± 1.49	--	--	--
	AIB, 25	106	6.6	69.65 ± 0.61	+8.35	41.14 ± 0.24	28.64
	AIB, 10	106	6.6	67.35 ± 2.89	+6.05	33.89 ± 0.03	28.69
	AIB, 5	106	6.6	63.75 ± 1.56	+2.45	24.73 ± 0.52	22.23
	AIB, 1	106	6.6	61.21 ± 0.20	-0.09*	12.86 ± 0.53	12.36
27	control, 0	124	36.7	140.35 ± 4.21	--	--	--
	AIB, 25	124	36.7	154.05 ± 0.59	+13.70	35.61 ± 0.80	23.11
	AIB, 10	124	36.7	152.92 ± 0.00	+12.57	26.26 ± 0.29	21.26
	AIB, 5	124	36.7	147.03 ± 2.99	+6.68	20.27 ± 0.27	17.77
	AIB, 1	124	36.7	146.73 ± 1.29	+6.37*	10.77 ± 0.03	10.27
34	control, 0	73	31.2	159.89 ± 1.38	--	--	--
	AIB, 25	73	31.2	152.45 ± 4.79	-7.44*	36.46 ± 1.51	23.96
	AIB, 10	73	31.2	160.08 ± 3.29	+0.19*	24.74 ± 0.01	19.74
	AIB, 5	73	31.2	157.90 ± 3.22	-1.99*	20.56 ± 0.53	18.06
	AIB, 1	73	31.2	156.80 ± 4.53	-3.09*	8.15 ± 0.03	7.65

39	control, 0	77	33.8	108.09 ± 1.86	--	--	--
	AIB, 25	77	33.8	112.41 ± 3.51	+4.32*	36.64 ± 1.41	24.14
	AIB, 10	77	33.8	107.47 ± 2.60	-0.62*	30.53 ± 3.48	25.53
	AIB, 5	77	33.8	105.72 ± 1.14	-2.37*	22.42 ± 1.28	19.92
	AIB, 1	77	33.8	107.16 ± 2.68	-0.93*	9.93 ± 0.27	9.43
43	control. 0	120	35.9	122.95 ± 1.93	--	--	--
	PHE, 25	120	35.9	121.35 ± 3.51	-1.60*	28.77 ± 0.37	13.17
	PHE, 10	120	35.9	123.66 ± 2.94	-0.71*	19.15 ± 1.11	12.91
	PHE, 5	120	35.9	124.56 ± 1.94	+1.61*	14.09 ± 0.15	10.97
	PHE, 1	120	35.9	122.40 ± 3.85	-0.55*	6.49 ± 0.11	5.97

[1]All fluxes were measured for a 1 min incubation period at 37 C. Symbols used are the same as those in Table 1 and 2.

*Difference is not statistically significant.

has a very low affinity for K+, and therefore, when the intracellular K+ is lowered, K+ is less likely to displace Na+ from the carrier ion site. As a further test of this hypothesis it was necessary to determine whether an increase in K+ influx occurred during AIB uptake.

The data given in Table 6 were used to calculate the change in K+ influx from K+ efflux and measurements of net movement in essentially the same way as sodium efflux was computed above. In this instance the equations used are as follows (the symbols are the same as those explained in Part I above):

$$\text{Net} = ([K+]_i^t - [K+]_i^o) \times SI$$

Thus a positive net flux indicates an increase in intracellular K+ concentration.

$$\text{Net} = \text{Influx} - \text{Efflux}$$

$$\text{Influx} = \text{Net} + \text{Efflux}$$

$$\text{Change in Influx} = (\text{Influx}_T - \text{Influx}_C)$$

$$= (\text{Net}_T + \text{Efflux}_T) - (\text{Efflux}_C + \text{Net}_C)$$

rearranging:

$$\text{Change in Influx} = (\text{Net}_T - \text{Net}_C) + (\text{Efflux}_T - \text{Efflux}_C)$$

The changes in K+ influx so computed are compiled in Table 7. According to this method of computation, a positive value for the change in K+ influx denotes that K+ influx increased during the amino acid uptake. The results in Table 7 show that only very small changes occurred in the K+ influx during the uptake of either AIB or phenylalanine. In one-half of the test samples the presence of the amino acid was apparently accompanied by a slight increase in the K+ influx, whereas in the other half it was accompanied by a slight decrease. However, the computed changes in K+ influx were very small and a slight error in the determination of the intracellular K+ concentrations could have made a relatively large difference in the K+ influx computed. Therefore, I decided to

TABLE 7

CALCULATION OF CHANGE IN K+ INFLUX DURING THE UPTAKE OF AMINO ACID.[1]

(1) Exp. No.	(2) Amino Acid & Concentration	(3) $[K+]_i$		(4) Swelling Index[2]	(5) Net K+ Movement[3]	(6) Change in Net K+ Movement (Net - Control Net)	(7) Change in K+ Efflux[4]	(8) Change in K+ Influx[5] [(7) + (6)]
		"0 - time"	After 1 min					
	mM	meq/liter			μmoles/g dry wt-min			
25	control, 0	106.0	121.2	3.64	+55.33	--	--	--
	AIB, 25	106.0	119.9	3.51	+49.02	-6.31	+8.35	+3.55
	AIB, 10	106.0	119.9	3.48	+48.23	-7.10	+6.05	-1.05
	AIB, 5	106.0	119.6	3.61	+49.17	-6.16	+2.45	-3.71
	AIB, 1	106.0	120.7	3.61	+53.23	--2.10	-0.09	-2.19
27	control, 0	124.3	123.6	4.12	-2.88	--	--	--
	AIB, 25	124.3	120.7	3.95	-14.22	-11.34	+13.70	+2.36
	AIB, 10	124.3	120.9	4.15	-14.11	-11.23	+12.57	+1.34
	AIB, 5	124.3	122.4	4.19	-7.96	-5.08	+6.68	+1.60
	AIB, 1	124.3	122.1	4.25	-9.35	-6.47	+6.37	-0.10
34	control, 0	72.9	75.8	3.39	+9.83	--	--	--
	AIB, 25	72.9	76.4	3.24	+11.34	+1.51	-7.44	-5.93
	AIB, 10	72.9	75.7	3.43	+9.72	-0.11	+0.19	+0.08
	AIB, 5	72.9	76.1	3.44	+11.03	+1.20	-1.99	-0.79
	AIB, 1	72.9	77.1	3.41	+14.31	+4.48	-3.09	+1.39

39	control, 0	76.7	82.3	3.29	+18.42	--	--	--
	AIB, 25	76.7	82.0	3.27	+17.21	-1.21	+4.32	+3.11
	AIB, 10	76.7	81.9	3.24	+16.76	-1.66	-0.62	-2.28
	AIB, 5	76.7	82.7	3.28	+19.63	+1.21	-2.37	-1.16
	AIB, 1	76.7	82.6	3.32	+19.61	+1.19	-0.93	-0.26
43	control, 0	120.3	117.6	3.59	-9.69	--	--	--
	PHE, 25	120.3	119.2	3.37	-3.57	+6.12	-1.60	+4.52
	PHE, 10	120.3	118.2	3.55	-10.40	-0.71	-0.71	+1.42
	PHE, 5	120.3	117.9	3.62	-11.92	-2.23	+1.61	-0.62
	PHE, 1	120.3	118.2	3.67	-10.24	-0.55	-0.55	+1.10

[1]Data are from the same experiments as reported in Table 6. Incubations were for 1 min at 37 C.

[2]Grams intracellular water divided by the grams dry wt of the pellet.

[3]A positive value indicates a net gain of K+ over the 1 min of incubation.

[4]Taken from Table 6, column 6.

[5]A positive value indicates that there was a greater influx in the sample than in the control during the 1 min incubation period.

measure the change in K+ influx directly by observing the rate of uptake of ^{42}K in the presence and absence of AIB uptake. The results of the experiment are given in Table 8, and confirm the results of the computed changes in K+ influx, i.e., AIB uptake produced no statistically significant increase in the K+ influx.

In summary, there is associated with AIB uptake the following ion movements: (1) an increased influx of Na+ equal to the saturable portion of the AIB influx, (2) an increase in the Na+ efflux of up to 50% of the saturable AIB influx, (3) an increase in the K+ efflux of up to 60% of the saturable AIB influx, (4) no significant increase in the K+ influx. If the K+ is replacing Na+ on the amino acid carrier at the intracellular side of the membrane, and if the carrier is operating in an electrically neutral fashion, i.e., not producing either a net influx or efflux of cation, then one would expect that the sum of the increase in K+ efflux and the increase in Na+ efflux would be equal to the saturable AIB influx.

B. Simultaneous Determination of Na+ and K+ Movements Associated with AIB Uptake.

I tested the above hypothesis by designing a triple-label experiment in which three parameters were simultaneously measured: (1) the influx of AIB (using tritium label), (2) the influx of Na+ (using $^{22}Na+$), and (3) the efflux of K+ (by measuring the efflux of preloaded ^{42}K). These experiments were conducted with fewer sample tubes per experiment (i.e., only two instead of four AIB concentrations were tested per experiment) in order to reduce the handling time, and in order to increase the accuracy of the determination of both the ion tracer measurements and the intracellular ion concentrations.

The same methods and equations as presented earlier were then used to calculate the changes in Na+ efflux (Table 9) and the changes in K+ influx (Table 10). As before the uptake of AIB was accompanied by both an increase in the Na+ influx (Table 9, column 7) and an increase in the Na+ efflux (Column 8). Also as before, Table 10 shows that with

TABLE 8

COMPARISON OF THE CHNAGE IN K+ INFLUX TO THE SATURABLE PORTION OF AMINO ACID INFLUX.[1]

(1) Exp. no.	(2) Amino Acid & Concentration	(3) $[K+]_i$	(4) $[K+]_e$	(5) K+ Influx	(6) Change in K+ Influx (Influx - Control Influx)	(7) Total Amino Acid Influx	(8) Saturable Amino Acid Influx
	mM	meq/liter		μmoles/g dry wt/min			
42	control, 0	118	32.4	28.60 ± 2.30	--	--	--
	AIB, 25	118	32.4	25.32 ± 0.43	-3.28*	41.10 ± 2.48	28.60
	AIB, 10	118	32.4	27.44 ± 0.97	-1.16*	30.53 ± 1.34	25.53
	AIB, 5	118	32.4	27.10 ± 2.02	-1.50*	26.07 ± 0.91	23.57
	AIB, 1	118	32.4	26.16 ± 0.18	-2.44*	11.21 ± 0.71	10.71

[1]Fluxes were measured over 1 min of incubation at 37 C.

*Difference is not statistically significant.

TABLE 9

TRIPLE LABEL EXPERIMENTS. CALCULATION OF THE CHANGE IN Na+ EFFLUX OCCURRING DURING AIB UPTAKE.[1]

(1) Exp. No.	(2) Amino Acid & Concentration	(3) $[Na^+]_i$		(4) Swelling Index	(5) Net Na+ Movement	(6) Change in Net Na+ Movement (Net - control Net)	(7) Change in Na+ Influx[2]	(8) Change in Na+ Efflux[3] [(7) - (6)]
		"0-time"	After 1 min					
	mM	meq/liter			μmoles/g dry wt-min			
41	control, 0	40.9	40.0	3.62	-3.26	--	--	--
	AIB, 25	40.9	42.4	3.43	+5.15	+8.41	+22.22	+13.81
	AIB, 5	40.9	42.4	3.66	+5.49	+8.75	+16.92	+8.17
45	control, 0	41.5	37.4	3.71	-15.21	--	--	--
	AIB, 10	41.5	40.9	3.73	-2.24	+12.97	+23.39	+10.42
	AIB, 1	41.5	37.7	3.80	-14.44	+0.77	+8.94	+8.17

[1]All incubations were for 1 min at 37 C. All sign conventions and calculations are the same as those used for Table 3.

[2]The change in Na+ influx was calculated as the difference of the control and test sample influxes of Na+ both of which were approximated with the use of $^{22}Na^+$.

[3]As in Table 3, a positive value for this parameter indicates that there is an increase in Na+ efflux in the test sample compared with the control.

TABLE 10

TRIPLE LABEL EXPERIMENTS. CALCULATION OF THE CHANGE IN K+ INFLUX OCCURRING DURING AIB UPTAKE.[1]

(1) Exp. No.	(2) Amino Acid & Concentration	(3) $[K+]_i$		(4) Swelling Index	(5) Net K+ Movement	(6) Change in Net K+ Movement (Net - Control Net)	(7) Change in K+ Efflux[2]	(8) Change in K+ Influx[3] [(6) + (7)]
		"0 - time"	After 1 min					
	mM	meq/liter			μmoles/g dry wt-min			
41	control, 0	121.6	118.6	3.62	-10.86	--	--	--
	AIB, 25	121.6	115.9	3.43	-19.55	-8.69	+10.08	+1.39
	AIB, 5	121.6	116.3	3.66	-19.40	-8.54	+9.45	-0.91
45	control, 0	118.4	120.8	3.71	+8.90	--	--	--
	AIB, 10	118.4	118.4	3.73	0.00	-8.90	+8.41	-0.49
	AIB, 1	118.4	120.5	3.80	+7.98	-0.92	+2.91	+1.99

[1]All incubations were for 1 min at 37 C. All sign conventions and calculations are the same as those in Table 7.

[2]The change in K+ efflux was calculated as the difference of the control and test sample K+ effluxes both of which were measured by the efflux of preloaded ^{42}K.

[3]As in Table 7, a positive value for this parameter indicates that there is an increase in K+ efflux in the test sample compared to the control sample.

AIB uptake there was an increase in K+ efflux (column 7) but no change in K+ influx (column 8). These results were then compiled in the bar graph of Figure 6. For each AIB concentration used: the first bar of the group represents the saturable portion of the AIB influx; the second bar is the measured increase in Na+ influx; while the third bar is the sum of the calculated increase in Na+ efflux and the measured increase in K+ efflux. The significant finding here is the near equality of these three parameters over the four AIB concentrations used. That is, it appears that the saturable AIB influx is accompanied by both an equimolar increase in the total cation (Na+ and K+) efflux as well as an equimolar increase in the Na+ influx.

As a further test of this apparent electroneutrality of the amino acid carrier operation, I compared the concentrative ability of cells incubated in normal Na-KRP and cells incubated in the same medium with all the chloride replaced by sulphate. Sulphate replacement of the extracellular chloride leaves only $SO_4^{=}$ and the various phosphates as extracellular anions, and these anions have all been shown to penetrate the Ehrlich cell only very slowly (Heinz and Mariani, 1957; Aull, 1967; Levinson, 1966). If the A amino acid carrier were producing any net cation movement, then in a normal medium Cl- might neutralize the electrogenic effect of the carrier operation by moving in the same direction as the net cation movement. However, in the sulphate medium there is no readily penetrating anion to accompany a net cation movement. In this situation, if only 1% of the increase in Na+ influx occurring with AIB uptake were unmatched by an efflux of cation, then I have calculated[1] that the resultant electrogenic effect would produce a transmembrane potential of over 1200 mV within one minute of AIB uptake from a 25 mM extracellular concentration. The development of such a transmembrane potential, besides being very improbable, would oppose the directional operation of an electrogenic carrier, and consequently it might be expected that sulphate replacement for Cl- would decrease the concentrative

[1]Assumes a membrane capacity of 1μF/cm^2.

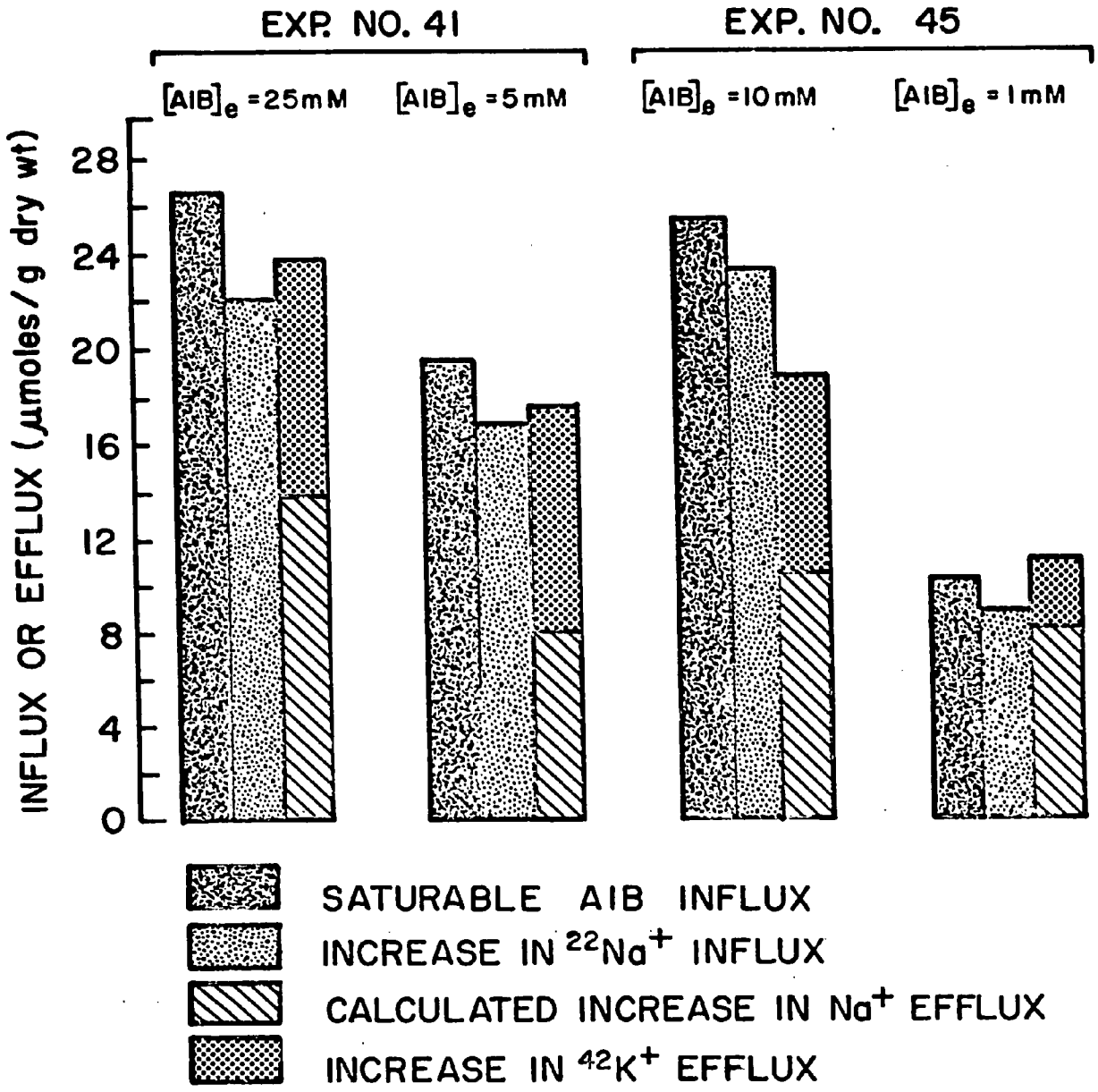


Figure 6.-- Triple-label experiments: Comparison of the Na+ dependent AIB influx, the increase in Na+ influx, and the sum of the increased K+ and Na+ effluxes. Fluxes were measured for incubation periods of 1 min at 37 C at the initial AIB concentrations designated. Data are from Tables 9 and 10.

ability of such a carrier. However, the results in Table 11 show no significant effect due to sulphate replacement except for the longest incubation time, 30 min. A sulphate effect is expected after longer incubations since Aull (1967) has shown that sulphate substitution for Cl- causes a loss of 20% of the intracellular K+ within 15 min and that there are potential shifts with longer sulfate incubations. Furthermore, Adrian and Slayman (1966) report that muscle incubated in sulphate shows significant membrane deterioration in less than one hour. The results of these sulphate replacement experiments cannot conclusively demonstrate that the carrier is operating in an electroneutral fashion. Although all rapidly penetrating anions have been removed from the extracellular medium, it is still possible that one of the divalent cations is moving as a counterion to a net flux of monovalent cation. These experiments at least make it extremely unlikely that any anion is moving as the counterion to an electrogenic amino acid carrier.

C. Discussion: Na+ and K+ Movements Associated with AIB Influx.

The results presented above clearly show that when the intracellular K+ concentration is at 'normal' high levels, there is an increase in K+ efflux associated with the uptake of AIB but not with the uptake of L-phenylalanine. Moreover, the triple label experiments with these cells have shown that the sum of this increase in K+ efflux and the calculated increase in Na+ efflux is approximately equal to the increase in sodium influx. In other words, AIB influx is associated with an equimolar increase in the total cation efflux (the sum of the increase in Na+ and K+ efflux) as well as an equimolar increase in Na+ influx.

However, when the intracellular K+ was lowered to levels approximately 60% of normal, the increase in K+ efflux was eliminated. Crane (1965) has presented evidence that K+ does replace Na+ from its carrier site when the carrier complex is on the intracellular side of the membrane. If K+ had a much higher K_m than Na+ for the carrier site, then it would be expected that, although K+ has a higher intracellular

TABLE 11

EFFECT OF SUBSTITUTING SULPHATE FOR CHLORIDE IN THE EXTRACELLULAR MEDIUM

Exp. No.	Incubat. Period	CONTROL		$SO_4^=$ SUBSTITUTION	
		$[Cl^-]_e$	AIB (C_i/C_e)	$[Cl^-]_e$	AIB (C_i/C_e)
	min	meq/liter		meq/liter	
35	1	126.0	0.77 ± 0.02	1.76	0.75 ± 0.03*
	5	128.9	2.39 ± 0.10	1.88	2.16 ± 0.11*
	30	133.0	8.12 ± 0.06	1.99	5.02 ± 0.28#
38	1	125.1	1.07 ± 0.06	2.64	1.09 ± 0.01*
	2	125.3	1.54 ± 0.06	2.93	1.54 ± 0.13*
	5	126.2	1.94 ± 0.08	3.41	2.08 ± 0.09*

*Not significantly different from control distribution ratio by 'Student's' t-test.

#Significantly different from control, $P < 0.02$.

concentration than Na+, only a portion of the carrier-bound Na+ would be replaced by K+. Furthermore, if intracellular K+ is partially replaced by Na+ through a treatment such as cold shock, then the increase in K+ efflux could be reduced to such an extent as to be unobservable. If it is assumed that the maximal increase in K+ efflux theoretically attainable is equal to the increase in Na+ influx, i.e., complete K+ replacement of Na+ on the carrier, then the K_m for K+ on the carrier would be on the order of 110-130 mM, or almost 10 times the K_m for Na+.

The calculation of K+ influx, and the direct measurement of this parameter, show that there is no increase during AIB uptake. Thus, AIB uptake while increasing the efflux of both Na+ and K+, increases only the influx of Na+. This would be another consequence of the Km for K+ being much higher than for Na+. Under these circumstances even a small extracellular Na+ concentration would exclude K+ from the carrier on the extracellular side of the membrane.

This asymmetry of the AIB effect of Na+ and K+ movements thus results in a greater increase in Na+ influx than in K+ efflux. Harris and Maizels (1951) have suggested that most sodium and potassium movements are reciprocal, that is, in a 1:1 ratio. However, Maizels et al. (1958a, b) and Hempling (1958, 1959) have since shown that such 1:1 movements are the exception rather than the rule in the Ehrlich ascites cell. In contradiction to my results, Hempling and Hare (1961) have reported that both the influx and efflux of K+ are increased in the presence of a glycine gradient. However, in their experiments K+ flux measurements were made over 10-min time periods during which the glycine distribution ratios became quite large. This glycine accumulation was accompanied by significant swelling of the cells, which by increasing the cell surface area would undoubtedly have increased potassium fluxes in both directions. In the experiments reported here, fluxes were measured over incubation periods of only one minute during which time no significant cell swelling or shrinking was observed.

The interpretation of the movements of ions associated with AIB uptake as being an equimolar Na+ influx, and an equimolar Na+ plus K+ efflux implies that the carrier is operating in an electrically neutral fashion. The experiments in which the only freely permeable anion, Cl-, was replaced by the almost non-penetrating anion, sulfate, also provide some support for this hypothesis. It seems unreasonable that a medium containing no rapidly penetrating anion could support the same rate of operation of a transport process producing net cation movement as a medium containing Cl-. This agrees with the findings of Bihler and Crane (1961, 1962) who replaced medium Cl- with several non-penetrating anions (including sulfate) and found that sugar uptake was not affected. The same was found by Vogel and Kröger (1966) for the sodium-dependent transport of PAH by the kidney. On the other hand, Vidaver (1964c) found that the non-penetrating anion mucate did decrease the rate of glycine transport by the pigeon red blood cell, and he used this finding to argue that the carrier was operating electrogenically. However, this transport system was also shown to have a 2:1 stoichiometry of sodium:glycine entry on the carrier indicating an obvious dissimilarity to the A system carrier of the Ehrlich Cell.

The association of AIB and ion movements thus appeared to be in accordance with the predictions of the sodium-potassium gradient hypothesis of Crane. However, certain of the findings presented in Part I tended to contradict the dependence of the AIB uptake rate on the sodium gradient.

Part III. An Examination of the Ion Gradient Hypothesis for Amino Acid Transport.

A. AIB Efflux with a Reversed Sodium Gradient.

Perhaps the most convincing support for the hypothesis that the sodium gradient provides the energy necessary for sugar uptake by the intestinal mucosa was the demonstration by Crane (1964) that an uphill efflux of preloaded sugar could be produced by reversal of the sodium gradient. Therefore, in order to test the applicability of this hypothesis to amino acid transport in the Ehrlich ascites cell, I attempted to reproduce essentially the same experiment in this system.

The cells were incubated in a Na-KRP (Na+-phosphates) medium containing 30 mM AIB at 37 C for 30 min. After this length of time the intracellular AIB concentration was found to be 45-50 mM. The suspension was then divided into three parts which were centrifuged and washed once, one part with Na-KRP (Na+-phosphates), one part with Ch-KRP, and one part with K+-KRP. Each part was then resuspended in the same media as was used for the wash procedure. The three suspensions were incubated for 5 min at 37 C, and subsequent sampling procedures were routine as described in METHODS. Thus the amount of AIB efflux was determined in three situations: (1) with a normal sodium and potassium gradient (suspension in Na-KRP), (2) with the sodium gradient reversed (suspension in Ch-KRP), and (3) with both gradients reversed (suspension in K-KRP). The results of this experiment are given in Table 12. In none of the three experiments was an active efflux of the AIB obtained (i.e., all distribution ratios remained greater than 1.0). Efflux was faster in those samples having a reversed sodium gradient, but the efflux was fastest when both the sodium and potassium gradients were reversed; however, this might be expected since in those suspensions with no extracellular Na+ there would be less reentry of the effluxed AIB. Furthermore, in the K+ medium there was considerable swelling of the cells (the swelling index reached 5.0), which

TABLE 12

DISTRIBUTION RATIOS FOR CELLS PRELOADED WITH AIB AND THEN INCUBATED IN DIFFERENT MEDIA.[1]

Na+ MEDIUM[2]			CHOLINE MEDIUM[3]			K+ MEDIUM[4]		
AIB (C_i/C_e)	$[Na^+]_i$	$[K^+]_i$	AIB (C_i/C_e)	$[Na^+]_i$	$[K^+]_i$	AIB (C_i/C_e)	$[Na^+]_i$	$[K^+]_i$
	meq/liter			meq/liter			meq/liter	
22.45	50.8	99.1	17.79	35.1	113.0	15.14	29.0	116.4
22.60	49.0	100.1	18.34	34.2	110.7	15.46	26.5	108.8
22.37	52.0	101.1	18.38	35.5	109.7	15.19	28.6	115.9
22.47 ± 0.12*			18.17 ± 0.33*			15.26 ± 0.17*		

[1]Cells were preloaded to an intracellular concentration of approximately 50 mM in Na-KRP, washed free of extracellular AIB, and then incubated in the various media for 5 min at 37 C.

[2]$[Na^+]_e$ = 150.0, $[K^+]_e$ = 7.0.

[3]$[Na^+]_e$ = 18.2, $[K^+]_e$ = 21.3.

[4]$[Na^+]_e$ = 16.5, $[K^+]_e$ = 137.3.

*'Student's' t-test shows that any two of the three means are significantly different with $P < 0.01$.

indicated an increase in the membrane surface area available for AIB efflux.

At this point I decided to repeat the same experiment using a time study of the efflux of AIB into the Na-KRP and Ch-KRP media only. The results of this experiment are given in Table 13. Once again the AIB distribution ratios did not even approach 1.0, although the incubation period was carried out to one hour. As before the AIB ratio did fall more rapidly when the Na+ gradient was reversed (i.e., in Ch-KRP), but, as before, this might very well be attributable to a greater reentry of the AIB from the Na-KRP media as compared to the Ch-KRP media. Secondly, after 60 min of incubation the intracellular sodium concentration of the cells in the Ch-KRP was approximately the same as the extracellular sodium concentration. It appeared that a concentrative AIB efflux could not be obtained, at least in the time intervals examined. In view of these ambiguous findings, I decided to alter the nature of these experiments as described in the next section.

B. AIB Accumulation with Reversed Ion Gradients.

The above experiments were designed to test the hypothesis that the sodium gradient did drive the accumulation of AIB, and, had an active efflux of AIB been found, it would have provided strong support for the hypothesis. However, the failure to find it could not be used to refute the hypothesis since the incubation times might have been too short, or the reversed ion gradients might have dissipated too rapidly to produce an active efflux. Therefore, I decided to reexamine the issue by reversing the hypothesis tested; that is, I assumed the sodium gradient or a combination of the sodium and potassium gradients did not provide all the energy necessary for the accumulation of AIB. The critical test of this hypothesis was then whether the cell could accumulate AIB in the face of a reversed sodium gradient or a combination of a reversed sodium and potassium gradient.

To do this, cells were prepared by a cold shock procedure and

TABLE 13

TIME STUDY OF DISTRIBUTION RATIOS FOR CELLS PRELOADED WITH AIB AND THEN INCUBATED IN A Na+ OR CHOLINE MEDIUM.[1]

Incubation Period	Na+ MEDIUM[2]			CHOLINE MEDIUM[3]		
	AIB (C_i/C_e)	$[Na+]_i$	$[K+]_i$	AIB (C_i/C_e)	$[Na+]_i$	$[K+]_i$
min		meq/liter			meq/liter	
5	54.37 50.34 52.36 ± 2.85	47.8 50.9	93.1 92.2	38.35 45.59 41.97 ± 5.12#	30.9 30.3	121.7 118.8
10	32.95 32.20 32.58 ± 0.53	43.5 44.8	91.1 88.1	27.53 25.36 26.44 ± 1.53*	20.7 20.6	126.2 129.3
30	20.66 23.42 22.04 ± 1.95	68.3 71.0	85.2 85.9	11.71 11.43 11.57 ± 0.20*	12.0 13.5	142.2 144.2
60	13.96 14.36 14.16 ± 0.28	66.2 76.6	78.7 84.8	7.01 7.53 7.27 ± 0.37*	11.8 9.6	150.0 147.6

[1]Cells were preloaded with AIB to an intracellular concentration of approx 80 mM and then washed and resuspended in one of the above media. Samples were then taken after the given incubation periods.

[2]$[Na+]_e$ = 122.2, $[K+]_e$ = 32.3 mM. [3]$[Na+]_e$ = 10.2 mM, $[K+]_e$ = 30.9 mM.

*'Student's' t-test shows value is significantly different than control, $P < 0.05$.

with the use of ouabain (2.5×10^{-4} M) in the bathing medium. Such preparative procedures generally raised intracellular sodium concentrations to 70-90 meq/liter and reduced potassium to 65-85 meq/liter. The cells, prepared in this manner, were then placed in a medium made up of approximately one-third Na-KRP and two-thirds K-KRP or Ch-KRP, which provided for extracellular sodium concentrations in the range of 35-50 meq/liter. These suspensions were then incubated for various time intervals at 37 C with AIB present. Table 14 presents the results of these experiments all of which show that AIB is accumulated to distribution ratios greater than 1.0 in the presence of a reversed sodium gradient. Of particular interest is experiment No. 22 in which both the sodium and potassium gradients were reversed (by the use of K+-KRP) throughout at least the first five minutes of the incubation period, and yet, the AIB distribution ratio reached 1.5 in this interval. The results of this experiment are graphically illustrated in Figure 7. (Note that as with AIB a Na+ or K+ distribution ratio greater than 1.0 indicates that the ion has a higher intracellular concentration than extracellular.) Experiment No. 24 (Table 14, Fig. 8) provides even more dramatic evidence that AIB can be accumulated in the presence of a reversed Na+ gradient. In this experiment the intracellular sodium concentration was greater than the extracellular for about 20 min of the incubation period, while in the same time interval the AIB distribution ratio reached 5.0.

C. Steady-State Flux Analysis.

In consideration of the evidence above which shows that AIB can be accumulated actively in spite of the reversal of both the Na+ and K+ gradients, I decided to test this failure of the ion gradient hypothesis in an experiment similar to that of Hempling and Hare (1961). As described in the INTRODUCTION, these authors measured the steady-state fluxes of glycine and K+ (using ^{42}K), and, knowing the gradients of both substances, they calculated that the maximal rate of expenditure of free

TABLE 14

TIME STUDY OF AIB DISTRIBUTION RATIOS FOR CELLS WITH A REVERSED Na+ GRADIENT.[1]

Exp. No.	Incubat. Period	Initial $[AIB]_e$	AIB Distribution Ratio	[Na+]		[K+]	
				Intracell.	Extracell.	Intracell.	Extracell.
	min	mM	(C_i/C_e)	meq/liter			
22#	2	10.0	1.00	56.9	49.0	93.6	109.8
	5	10.0	1.48	51.4	47.6	103.4	110.7
	10	10.0	1.85	37.9	50.7	138.1	107.5
	30	10.0	2.66	19.0	53.7	143.4	102.4
24*	2	2.0	2.40	84.7	35.3	89.9	26.8
	5	2.0	3.38	74.0	36.2	100.6	26.8
	10	2.0	4.18	58.8	37.4	102.6	26.8
	30	2.0	5.53	31.7	40.2	124.8	26.8
26*	2	10.5	1.02	86.2	34.6	73.0	30.0
	5	10.5	1.38	79.3	36.0	80.8	29.8
	10	10.5	1.75	69.3	36.2	83.4	28.5
	30	10.5	2.46	46.7	38.8	98.6	28.6

[1]Cells used were prepared by the cold shock procedure and ouabain was present at 2.5×10^{-4} M throughout all procedures. Incubation was at 37 C.

#Na-KRP was partially replaced by K-KRP.

*Na-KRP was partially replaced by Ch-KRP.

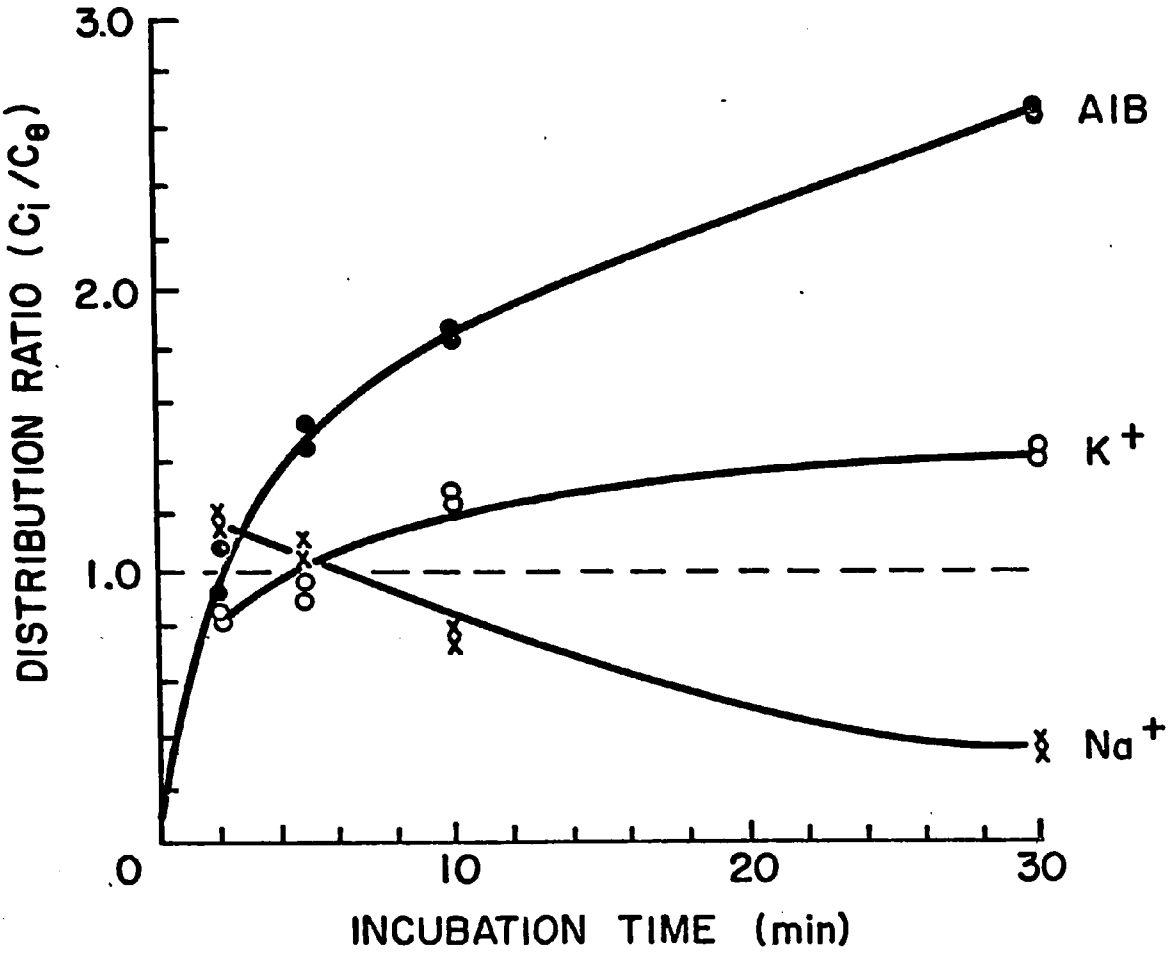


Figure 7.-- Time study of the distribution ratio of AIB during a period in which the Na+ and K+ gradients were reversed. The initial extracellular AIB concentration was 10 mM; incubation temperature was 37 C. Data are from Table 14.

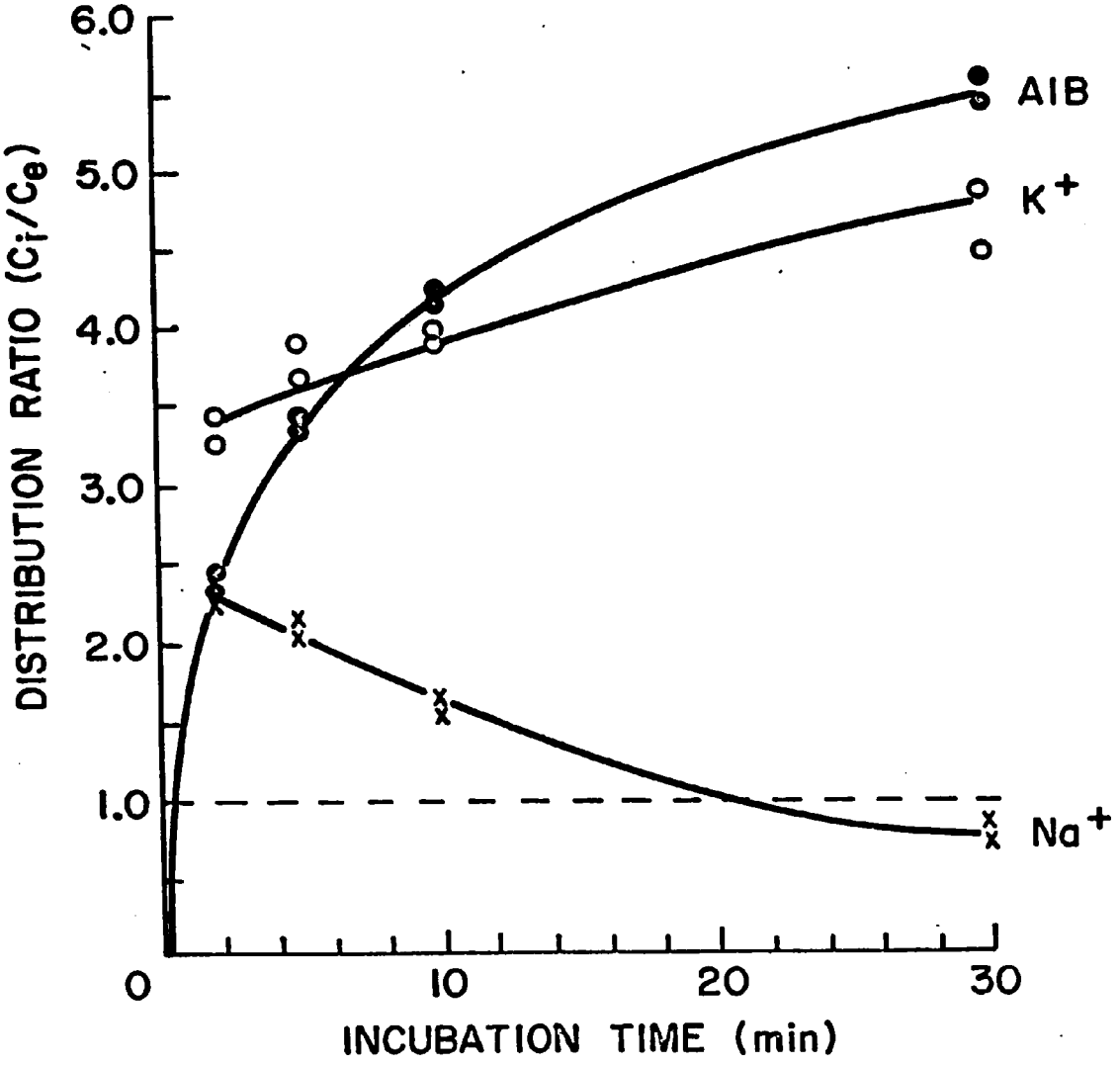


Figure 8.-- Time study of the distribution ratio of AIB during a period in which the Na+ gradient was reversed. The initial extracellular AIB concentration was 2 mM; the incubation temperature was 37 C. Data are from Table 14.

energy from K+ gradient was less than the minimal rate of expenditure of free energy for maintenance of the glycine gradient. This experiment provided the first direct evidence against the potassium gradient hypothesis of amino acid transport. Therefore, I felt that it would be valuable to repeat this experiment in a situation in which the sodium gradient was simultaneously reversed.

The cells were prepared with the cold shock procedure, and ouabain was present throughout all procedures at 5.0×10^{-4} M in order to maintain intracellular Na+ at as high a concentration as possible. The cells were then incubated in a medium of one-third Na-KRP and two-thirds Ch-KRP with AIB present at an initial extracellular concentration of 2 mM. Two samples of the suspension were removed after 60 min and after 74 min of incubation for the determination of AIB (measured by tritium) and K+ distribution ratios. Since these parameters were essentially the same after 60 and after 74 min, it was assumed that a steady-state condition existed. Therefore, 74 min after beginning the incubation 0.1 ml of solution containing the AIB-^{3}H and ^{42}K were introduced into the remaining flasks (up until this point these flasks had been incubated with AIB but no label). The incubation was stopped at 75 min total incubation and subsequent analysis was conducted as described in METHODS. It was thus possible to compute the steady-state fluxes of both AIB and K+ from the amounts of intracellular tracers and the extracellular specific activity. However, in order to calculate the energy available in the K+ gradient it was necessary to have an estimate of the transmembrane potential.

Hempling and Hare (1961) in their calculations assumed a transmembrane potential of -11 mV (inside relative to outside), which they derived by assuming Cl- was passively distributed, and then computing the Cl- equilibrium potential according to the Nernst equation. Hempling and Kromphardt (1965) and Aull and Hempling (1967) have since shown that only 60-70% of the intracellular Cl- is exchangeable, which would imply a higher Cl- equilibrium potential. However, Aull (1967) has

actually measured the transmembrane potential in the Ehrlich cell by micropuncture as an average -11.2 mV, and she contends that the potential is a combination of both the Cl- and Na+ equilibrium potentials. According to Aull, replacement of extracellular Na+ by the non-penetrating cation choline would tend to increase the negativity of the transmembrane potential so that it would approximate the chloride equilibrium potential calculated on the basis of 70% non-exchangeable Cl-. In order to estimate the chloride equilibrium potential under the conditions used in my experiments, I measured the intracellular and extracellular chloride concentrations in the steady-state samples (Table 15). As might be expected the presence of the non-penetrating extracellular cation (choline) resulted in some shifting of intracellular chloride to the extracellular medium, thereby increasing the negativity of the chloride equilibrium potential. Since it is expected that choline when substituted for Na+ will increase the negativity of the transmembrane potential toward the chloride equilibrium potential, the true transmembrane potential for these cells must lie between the -11 mV measured for normal cells and the -37 mV calculated for the maximal chloride equilibrium potential (Table 15). For the purposes of my calculations, I have chosen the least upper bound of this range (-11 mV) for the transmembrane potential as this provides for an overestimate of the free energy in the K+ gradient. Using precisely the same equations and methods as Hempling and Hare (1961, appendix), I have calculated (Table 16) that the rate of expenditure of free energy necessary for the maintenance of the AIB gradient (W_{AIB}) was greater than the rate of expenditure of free energy from the K+ gradient ($-W_K$). Measurement of intracellular and extracellular sodium showed that, in the same sample suspensions, extracellular sodium was in the range 35-38 meq/liter while the intracellular sodium was in the range 40-47 meq/liter. Thus, this experiment provides evidence that there is insufficient energy in the K+ gradient to account for the AIB gradient observed. But furthermore, since the sodium gradient was simultaneously reversed, neither the sodium, nor

the potassium, nor even the sum of both gradients could account for the energy expenditure necessary to maintain the observed AIB gradient.

TABLE 15

Experiment No. 44. CALCULATION OF CHLORIDE EQUILIBRIUM POTENTIALS.

Sample No.	[Cl-] Intra.	[Cl-] Extra.	E_{Cl} [1]	$E_{Cl'}$ [2]
	meq/liter		millivolts	
7	43.80	125.34	-28.0	-37.5
8	43.92	125.28	-27.9	-37.4

[1] Chloride equilibrium potential, inside with respect to outside, according to the Nernst equation.

[2] Chloride equilibrium potential according to the method of Aull and Hempling (1967) (see text).

It should be noted that the $-W_K$ computed is an overestimate on two counts. First, it is an overestimate due to underestimating the negativity of the transmembrane potential as discussed above. Secondly, the measured K+ steady-state flux is an overestimate because the ^{42}K was known to enter the cells at a significant rate during the chilling and sampling period, whereas it would be expected that the chilling procedure would stop essentially all the AIB influx.

D. Discussion: Evidence Against an Ion Gradient Hypothesis.

The most serious objection that might be raised to the reversed gradient studies stems from the possibility of extreme heterogeneity in the cell population. Whittam (1964) has briefly discussed the problem of heterogeneity in red cell populations, and he has indicated that it is

TABLE 16

COMPARISON OF THE MINIMAL RATE OF FREE ENERGY EXPENDITURE FOR THE AIB GRADIENT AND THE MAXIMAL RATE OF FREE ENERGY EXPENDITURE FROM THE K+ GRADIENT

Exp. No.	$[K+]_i$	$[K+]_e$	$[AIB]_i$	$[AIB]_e$	K+ Efflux	AIB Influx	W_{AIB}[2]	$-W_K$[3]
	meq/liter				μmoles/g dry wt-min		(cal/g dry wt-min) x 10^3	
40	123.7	31.8	7.49	1.77	18.20	21.70	19.30	10.85
	132.4	31.8	7.72	1.82	18.22	22.68	20.17	11.63
44	113.9	30.1	7.42	1.90	23.22	17.18	14.46	13.40
	113.9	30.2	7.82	1.95	22.36	19.32	16.53	12.90

[1]See text for detailed discussion of calculations involved.

[2]Minimal rate of expenditure of free energy for the AIB gradient.

[3]Maximal rate of expenditure of free energy from the K+ gradient assuming a membrane potential of -11 mV; see text.

a factor deserving of consideration (although it seldom is considered) in transport investigations. In the above experiments it is possible that after the cold shock procedure one fraction of the cells might rapidly lose the accumulated Na+ and gain K+, while another fraction might retain very high Na+ and low K+ concentrations. Then, if the former fraction with normally directed gradients were strongly accumulating AIB, the averaged results from the whole cell suspension might show an active transport of AIB and reversed ion gradients, whereas in reality only the cells with ion gradients of normal direction would be producing the AIB accumulation. However, the AIB uptakes given in Table 14 were all from media containing only 35-50 meq/liter of Na+, and yet the reported distribution ratios are not much lower than would be expected for cells with normal intracellular ion concentrations suspended in a medium with such a low Na+ concentration. Therefore, most of the cells must be contributing to the active AIB accumulation evidenced, and it would seem impossible that any heterogeneity could account for the results.

Furthermore, there is little reason to suspect any marked heterogeneity in these experiments. Cells prepared by the cold shock procedure did not appear to differ from those prepared by the room temperature procedure when examined under the microscope, and the proportion of "damaged" cells (those penetrated by trypan blue) was the same with both preparations. Jacquez (1966) has observed that there is a preponderance of abnormal cell types in the top cell layer of a centrifugal pellet; however, in these experiments the top cell layer was removed by suction pipette after each of the several preparative centrifugations.

Thus the results given above show that AIB can be actively accumulated in spite of a reversed sodium gradient. AIB distribution ratios of up to 5.0 were obtained under these conditions. Furthermore, there is definite evidence in the results above that the entire uptake of AIB cannot be energetically driven by a combination of the sodium and

potassium gradients. This evidence comes most directly from the observation of a concentrative uptake of AIB while both gradients were reversed (Fig. 7). This finding is strongly supported by the results of the steady-state flux experiments, which demonstrated that the maximal rate of free energy expenditure from the K+ gradient was less than the minimal rate of free energy expenditure necessary for the AIB gradient established. Since the Na+ gradient was simultaneously reversed in these experiments it would, if the Na+-K+ gradient hypothesis were valid in this system, tend only to increase the energetic requirement of the AIB gradient. Further evidence against the ion hypotheses is available by comparing the data of experiments 22 and 26 (Table 14). Although the direction of the potassium gradient is opposite in the two experiments and the sodium gradient is reversed in both, the time course of AIB uptake is approximately the same in both experiments.

These results show that neither the sodium gradient hypothesis proposed for amino acid uptake in the pigeon red blood cell (Vidaver, 1964a) nor the sodium-potassium gradient hypothesis for the intestinal transport of sugars (Crane, 1965) can be used to account for all the energy expenditure necessary for the active transport of AIB in the Ehrlich cell. Obviously these results do not contradict the validity of the hypotheses in the systems from which their supporting evidence is derived, but they do contradict the suggestion of Crane (1965) that ion gradients might be a universal source of energy for the active transport of non-electrolytes. Curiously in those experiments in which Crane (1964) showed an uphill efflux of sugar when the sodium gradient was reversed both anoxia and dinitro cresol were used. These conditions would suppress any operation of the carrier depending on direct linkage to cellular metabolism, a possibility which will be discussed later. Earlier, Kipnis and Parrish (1965) also stated that the sodium gradient hypothesis did not appear to be valid for the Na+-dependent transport of amino acids in skeletal muscle since they found that the uptake rate did not show the expected dependence on the intracellular Na+ concentration.

Part IV. Replacement of Extracellular Na+ by Other Monovalent Cations.

The requirement for Na+ in the transport of amino acids in the Ehrlich ascites cell appears to be quite analagous to that in other transport systems. This requirement has been found in several other systems to be very specific in that other monovalent cations cannot substitute for Na+. Earlier studies have shown that neither K+ nor choline could substitute for Na+ in amino acid transport in the Ehrlich cell (Christensen et al., 1952a; Christensen and Riggs, 1952). Consequently I felt it would be of value to determine if any of the other alkali metal ions (Li+, Cs+, or Rb+) could substitute for Na+. This information would at least provide another means of comparing this transport system with others.

In order to determine the ability of the other alkali metal ions to replace Na+, time studies of the distribution ratios of AIB (initially present at 2 mM) were conducted in the following media: Na-KRP, K-KRP, Cs-KRP, Li-KRP and Rb-KRP. As a comparison the same experiment was also conducted in Ch-KRP.

Figure 9 shows that, while Na-KRP supported an uptake of AIB sufficient to produce a distribution ratio of 24.0 within 30 min, substitution of the other alkali metal ions and choline gave at best a distribution ratio of less than 3.0 in the same time period. Potassium was observed to support the lowest AIB uptake, which was not even concentrative after 30 min of incubation. However, K+ was also observed to produce the greatest degree of cell swelling (the swelling index reached 6.9), followed by Rb+ (swelling index, 5.4). Choline gave a slight shrinkage compared to Na+ controls (swelling index, 3.2), whereas Li+, Na+ and Cs+ all gave the same swelling index range of 3.4-3.7. This induced me to replot the results of Figure 9 on the basis of total AIB uptake in μmoles/g dry wt over the incubation period. When this was done (Fig. 10) it was found that there was no significant difference in the AIB uptake rates from Cs-KRP, Rb-KRP or K-KRP, and a single line was used to indicate their time-course of uptake. Choline-KRP gave almost the same uptake rate as observed in the afore-mentioned media; however,

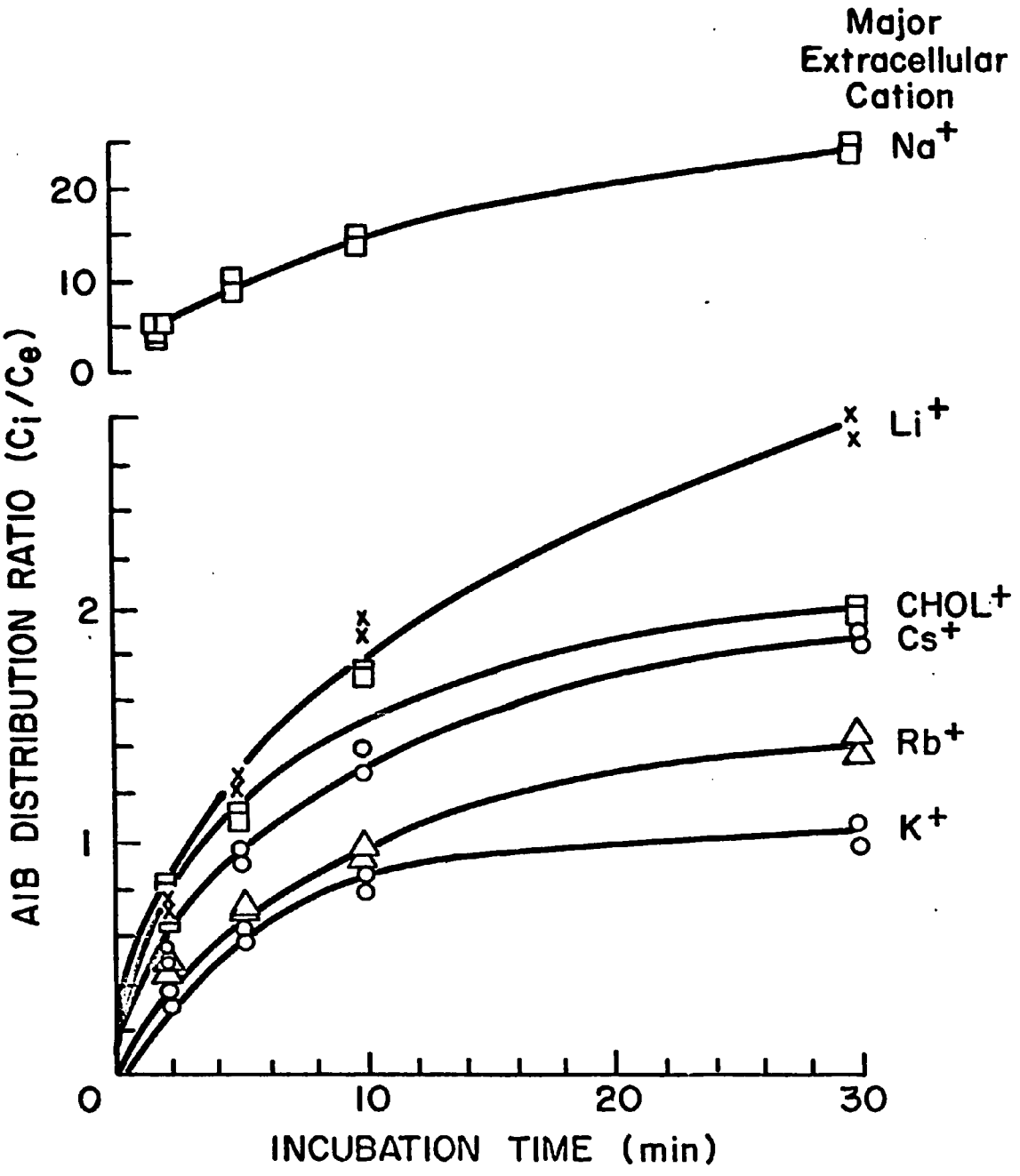


Figure 9.-- The effect of replacing Na+ by other alkali metal ions and choline on the distribution ratio of AIB. This time study was conducted for an initial extracellular AIB concentration of 2 mM at 37 C. Each curve is labeled according to the cation replacing Na+.

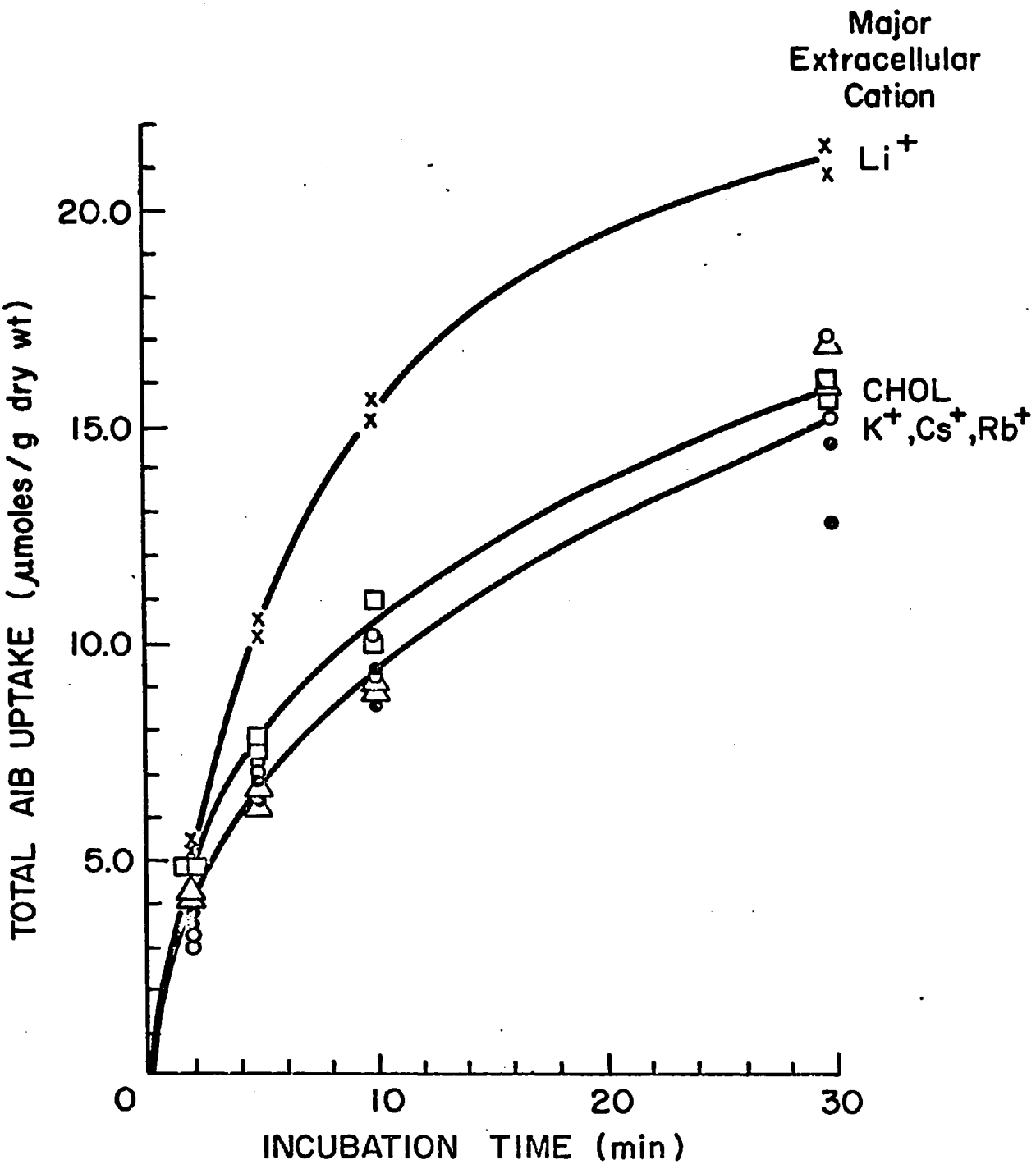


Figure 10.-- The effect of replacing Na+ by other alkali metal ions and choline on the total AIB uptake. Conditions and symbols are the same as in Fig. 9.

this time-course is shown as a separate line since the uptakes observed at the three lowest times were significantly different than those for the other three media at $P<0.05$. However, the uptake rate from Li-KRP was significantly higher than the uptake rates from the other three media or choline ($P < 0.005$ for the three longest incubation times, $P<0.01$ for the shortest period).

Several investigators of sugar transport in the gut have reported that replacement of Na+ by K+ is far more inhibitory to the transport process than replacement by choline or sucrose (Bosackova, 1963; Crane, 1965; Robinson, 1966). These investigators have interpreted this finding to indicate that K+ is combining with the carrier ion site and thereby greatly increasing the K_m for substrate. However, the results presented here suggest that the additional inhibition of transport evidenced in the presence of K+ may be a reflection of the considerable cell swelling evidenced with K+ rather than a reflection of a direct action of K+ on the carrier. In fact it is evident that all the substituting alkali metal ions (except Li+) and Choline give the same degree of inhibition of transport.

Li+ on the other hand is an exception. And, while it supports less than one-tenth the concentrative ability seen with Na-KRP, Li-KRP does support a significantly greater AIB uptake than any of the other alkali metal substitutions. This is further supported by the work of Faust et al. (1966), who report that Li+ may partially substitute for Na+ in the intestinal transport of D-Xylose, and by the work of Playoust and Isselbacher (1964), who find that Li+ can partially replace Na+ for the intestinal reabsorption of bile salts. The complete inability of any other alkali metal ions to replace Na+ is in accord with the same findings for the intestinal transport of sugars (Bihler and Crane, 1961; Boscakova, 1963).

GENERAL DISCUSSION

Perhaps the first question that should be raised about the results of this investigation is whether or not the reported measurements of changes in sodium and potassium movements are valid. I have already discussed the difficulty of measuring Na+ fluxes with tracer techniques, and this has been stressed previously by Maizels et al. (1958a, b). However, the important point here is that no attempt was made in these experiments to attach any significance to the absolute Na+ or K+ fluxes measured. Rather the important parameter was the change observed to occur in the Na+ or K+ flux during the accumulation of amino acid. If one considers the careful use of replicates, sampling time and sampling order in these experiments, then the observation that sodium influx and potassium efflux increased during AIB uptake is incontestable. The question then becomes whether the magnitude of these changes can be considered valid. The results reported in Tables 1 and 2 show that the changes in Na+ influxes were extremely consistent for replicates within any one experiment and for analagous samples in different experiments. Furthermore, the ratio of the increase in Na+ influx to the saturable AIB influx showed a 1:1 correspondence in such a variety of conditions as to rule out any merely fortuitous relationship. The results from measurements of the changes in K+ fluxes are more readily acceptable because of the lower permeability of K+ in the Ehrlich ascites cell. Measurements involving the use of ^{42}K were always made over a one-minute incubation period in which time the specific activity on the cold side was always less than 10% of that on the hot side. The use of a tracer to determine one-way fluxes under such conditions is completely conventional, and thus those changes in K+ fluxes which were directly measured can certainly be regarded as valid.

It must be admitted, however, that changes in the ion fluxes that were calculated on the basis of the net flux and the opposing flux are not that accurate. The determination of the change in net flux during amino

acid uptake depended on changes in intracellular ion concentrations of sometimes as little as 3%, a degree of accuracy that taxes the resolution of the flame photometer. But only one parameter depended entirely on such calculations, and that was the change in Na+ efflux. It is safe to say that this parameter did increase during AIB uptake but never to the extent of the increase in Na+ influx, and that it did not increase during the uptake of L-phenylalanine. This conclusion is supported in addition by the findings of the triple-label experiments (Fig. 6).

The next question of importance is whether the increased ion fluxes were a direct consequence of the operation of the sodium-dependent carrier or the result of a non-specific effect of the amino acid on the cell. Perhaps the best evidence in this regard is the fact that ion fluxes did not change during the uptake of phenylalanine, although this amino acid was present at the same extracellular concentrations as AIB, and was accumulated to approximately the same degree as AIB in the one minute incubation period. The only apparent difference between the uptake of AIB and the uptake of phenylalanine is the mediation of this uptake. There is little doubt that AIB is accumulated almost exclusively by action of the A carrier system. In referring to this carrier Christensen (1966) has stated, "... alpha-aminoisobutyric acid appears to be a nearly ideal model for one transport system occurring in the Ehrlich cell." And this is borne out by the results of Inui and Christensen (1966), which show that all the saturable uptake of AIB is sodium-dependent. From the results presented by Oxender and Christensen (1963) it is much more difficult to choose an amino acid that demonstrates uptake by the L carrier exclusively. Christensen (1966) has suggested tert-leucine may best fit this description; however, since tert-leucine was not available in ^{3}H-labeled form, I have used phenylalanine on the basis of the competition data presented by Oxender and Christensen (1963). These data show that, while phenylalanine uptake is strongly inhibited by the presence of other L-system amino acids, it is only slightly inhibited by the presence of AIB and other A system amino

acids. Thus, there is very strong evidence that the difference in the effect of AIB and phenylalanine on the Na+ and K+ fluxes lies entirely in the different modes by which they are taken up, i.e., operation of the A carrier but not the L carrier is accompanied by the increased ion movements.

Next, I wish to consider the implications of the observed changes in ion fluxes with regard to net ion movements. Christensen and Riggs (1952) and Christensen et al. (1952a, b) have reported that, during amino acid accumulation by the Ehrlich cell, there is a loss of cell K+ and a gain of Na+. My results would suggest that at least during the initial uptake of AIB there would be a net increase in Na+ influx since the increase in Na+ influx is greater than the increase in efflux. Also there would be an increase in K+ efflux since this increase is unmatched by an increased influx. Consequently, under normal conditions, the cells might initially gain Na+ and lose K+. However, such a change in net fluxes would probably cause an increased action of the sodium pump to prevent any large changes in the intracellular ion concentrations. This was demonstrated in the time-study experiments using Na-KRP (Fig. 9) by the observation that, although the intracellular Na+ rose and the K+ fell slightly in the first two minutes, no further significant change in these two concentrations was evidenced after longer time intervals. Jacquez[1] has examined the uptake of methionine in Ehrlich ascites cell suspensions incubated for up to one hour in Heinecke tubes, in which oxygen diffusion becomes a limiting factor, and in Erlenmeyer flasks, in which oxygen diffusion can occur readily. He found that cells in the latter vessels showed only slight ion shifts, whereas in the former case the cells showed a large loss of K+ and a gain of Na+ within 10 min. It seems in consideration of these results that the ion shifts observed by Christensen and his coworkers might represent deterioration of the cells over the long (1-2 hr) incubation periods used, rather than net ion movements associated with amino acid uptake.

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[1] Personal communication.

If the Na+ pump is activated by the increased Na+ influx and K+ efflux brought about by the operation of the A carrier, then the operation of this pump would be expected to increase the efflux of Na+ and the influx of K+ in order to counter any net ion shifts. Thus, the Na+ efflux observed with AIB uptake might merely be a reflection of the stimulation of the Na+ pump brought about by the increased Na+ influx. Here one is confronted with the problem of not having sufficiently accurate measurements of the change in Na+ efflux. The increased Na+ entry is matched by an equal increase in the total cation (Na+ and K+) efflux. This 1:1 relationship suggests that both the increased Na+ influx, and the increased cation efflux, are a result of the combination of Na+ and K+ with the A carrier. However, it might just as well be that the carrier has a negatively charged site on influx that binds the Na+, but that when intracellular this site is neutralized by some process which alters the carrier in order to return it to the extracellular side. Thus, the operation of the A carrier might still be electroneutral but increase the influx of Na+, which, in turn, would stimulate operation of the Na+ pump and cause an increase in Na+ efflux. This hypothesis is countered by the finding that K+ efflux is also increased with no concurrent change in K+ influx. An increased operation of the sodium pump would be expected to be accompanied by just the opposite K+ flux changes. Thus, while an increased operation of the Na+ pump very definitely must enter into the picture at longer time intervals it seems unlikely that it is contributing much to an increased Na+ efflux in the one-minute incubation periods used for the flux measurements.

The changes in Na+ and K+ fluxes I have observed support the theory of Crane (1965) that sodium combines with the carrier extracellularly in a 1:1 ratio with the transported non-electrolyte and then is replaced on the intracellular side of the membrane by K+, which effluxes with the carrier. However, my results show that K+ replaces only a fraction of the Na+ on the carrier. And, in contrast to Crane's theory, the kinetic analysis of my results shows that the primary effect of

removing Na+ from the medium is to decrease the carrier v_{max} not to increase its K_m for amino acid. Furthermore, when examining the change in Na+ influx, the primary effect of lowering the AIB concentration was to decrease the apparent v_{max} but not to raise the K_m for the associated sodium influx. It appears that in the Ehrlich cell both Na+ and amino acid are required to be bound to the A carrier for inward movement of the entire complex, but that the presence or absence of Na+ or amino acid on the carrier does not alter the affinity of the other entity for its carrier site. Crane interpreted his finding of a Na+ effect on the sugar K_m to result from an allosteric effect of the sodium, when at its own site, on the carrier sugar site. On the other hand, my results predict little or no allosteric effect for the A system carrier of the Ehrlich ascites cell.

In addition, the results of my studies on the effect of ion concentration gradients are at variance with any application of the sodium-potassium gradient hypothesis of Crane (1965) to amino acid transport in the Ehrlich cell. I have found the A carrier system of the Ehrlich cell can establish a very definite active transport of AIB in spite of a reversal of the sodium gradient. Furthermore, I have shown an active accumulation of AIB in a period in which both the sodium and potassium gradients were reversed, thereby providing direct evidence against the hypothesis in this system. Crane's best support for his hypothesis is derived from the finding of an active sugar efflux in the presence of a reversed sodium gradient. However, since these studies were done under complete metabolic inhibition, they cannot possibly establish the Na+ gradient as the sole energy source for sugar transport. I do not wish to take issue with the possibility that the ion gradients may provide some of the energy for amino acid transport. In fact, if the ions are combining with the carrier and are necessary for its translocation, then the normal ion gradients could very well provide some energy for the system. (In this respect it would be interesting to repeat the experiments of Crane in the Ehrlich cell, i.e., examine amino acid efflux in the presence of a

reversed sodium gradient and complete metabolic inhibition.) My main contention here, and it seems self-evident from the results, is that neither the sodium gradient nor the potassium gradient nor both gradients can be providing all the energy for amino acid transport by the A carrier in the Ehrlich ascites cell. It is not surprising to find that the energy in the ion gradients is insufficient for the energetic requirements of amino acid transport in this cell, because there is already good evidence that L carrier amino acids can be accumulated in the complete absence of extracellular sodium (Oxender and Christensen, 1963; Inui and Christensen, 1966). Also the Ehrlich cell shows a unique intensity of transport of several amino acids, and yet has a smaller Na+ gradient (50 mM intracellular to 150 extracellular) than most cells. From the above discussion one can see that, in addition to the ion gradients, this transport system must have some other linkage to cellular metabolism. It is the nature of this linkage that now assumes importance.

Perhaps, the most attractive intermediary for a linkage of energy to the transport process is the Na+-K+ sensitive ATPase discussed in the INTRODUCTION. This membrane ATPase has been shown to have the Na+ activation and narrow range of K+ sensitivity evidenced for most Na+- sensitive transport processes. Furthermore, Skou (1965) has observed that this ATPase shows almost the same sensitivity to Cs+ and Rb+ as it does to K+, while the effect of Li+ lies intermediate to that of K+ and that of Na+. This is the same set of findings as obtained when the effect of alkali metals was tested on amino acid transport. One possibility would be that the ATPase is part of the amino acid carrier, and, by binding K+ or Na+, it causes translocation of these ions. However, since there is an increase in Na+ influx and K+ efflux, this translocation would be in the opposite direction as was proposed for the ATPase action in the sodium pump (Post et al., 1960). The association of ATPase with any transport system seems to be merely by virtue of circumstantial evidence, and, in general,

ATPases have the disturbing history of being invoked to account for the control of almost any energy requiring process. In consideration of this, any model proposing the association of an ATPase with a transport process should await both the more discrete localization of the ATPase in the membrane and the further characterization of this enzyme.

I feel that the sodium requirement of amino acid transport in the Ehrlich ascites cell is a manifestation of the requirement for sodium as a cofactor on the amino acid carrier. In acting as a cofactor the sodium might be necessary either for some steric rearrangement of the carrier or for the enzymatic coupling of energy to the carrier. In either instance a lack of sodium might prevent carrier operation or at least the ability of the carrier to actively transport. This would result in a reduction of v_{max}, but it would not necessarily affect the K_m. Such a role for Na+ is less intrinsically interesting than its role in the sodium gradient theory. The results of this study show that linkage of metabolic energy to the active transport process is still an unsolved problem deserving of further investigation. In spite of this, the sodium requirement and ion fluxes observed for amino acid and other transport systems can still be used as a tool in the elucidation of carrier operation.

SUMMARY

1) The association of Na+ and K+ with amino acid transport in the Ehrlich ascites tumor cell was investigated.

2) During the uptake of alpha-aminoisobutyric acid (an A-system amino acid) the following changes in ion fluxes were observed to occur: (i) an increase in the influx of sodium (measured by ^{22}Na), (ii) an increase in Na+ efflux (calculated from the influx and net flux data), (iii) an increase in K+ efflux but not in K+ influx (both measured by ^{42}K).

3) The magnitude of the increase in Na+ influx was found to be equal to the saturable (sodium-dependent) portion of the AIB influx under a wide variety of conditions. Furthermore, these two quantities were approximately equal to the sum of the increased efflux of sodium and the increased efflux of potassium.

4) L-phenylalanine (an L-system amino acid) produced no significant changes in ion fluxes.

5) AIB was shown to be actively accumulated even when the sodium gradient was reversed or when both the sodium and potassium gradients were reversed.

6) Cs+, Rb+, K+ and choline were inable to substitute for the Na+ requirement of AIB transport in this system. However, Li+ was shown to have a slight ability to substitute for Na+.

7) These results were interpreted to show that ions were being moved by operation of the amino acid carrier. However, they provided concrete evidence that the ion gradients could not be providing all the energy necessary for amino acid active transport in the Ehrlich ascites tumor cell.

APPENDIX

Conversion Factors

The flux measurements in the results presented here are in terms of μmoles/g dry wt-min. These measurements can be converted to other commonly used units by multiplying the fluxes by the appropriate conversion factor given in Table 17. The conversion factors were observed to depend to some degree on the media used to suspend the cells, i.e., they depended on the degree of swelling or shrinkage produced by the media. Therefore, different conversion factors are given for each buffer solution used.

TABLE 17

CONVERSION OF UNITS

Buffer Medium[1]	$\frac{10^{-2} \text{ g dry wt-min}}{10^{7} \text{ cells-hr}}$	$\frac{10^{-5} \text{ g dry wt-min}}{\text{cm}^2\text{-min}}$	$\frac{\text{g dry wt-min}}{\text{kg intracell. } H_2O\text{-min}}$
Na+ (34)	8.638 ± 0.942	2.317 ± 0.140	2.697 ± 0.146
Chol+ (5)	8.191 ± 0.414	2.492 ± 0.068	3.148 ± 0.107
K+ (15)	11.256 ± 1.221	2.241 ± 0.290	1.938 ± 0.735
Li+ (1)	8.406	2.296	2.491
Cs+ (1)	7.614	2.342	2.952
Rb+ (1)	6.654	1.558	2.245

[1]Each medium includes KRP solutions made up with Na+ phosphates and with K+ phosphates. The number in parentheses is the number of experiments in which the particular buffer was used.

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