ABSTRACT

1. Methionine exchange diffusion is insensitive to Na\(^+\), K\(^+\), their gradients and prior depletion of cellular ATP.

2. In the absence of extracellular Na\(^+\), methionine uptake can be divided into two components: an initial rapid exchange between methionine and several endogenous amino acids and a slower net uptake inhibitable by \(\alpha\)-aminoisobutyric acid and L-alanine.

3. After depletion of endogenous ATP and amino acids, carrier mediated methionine uptake from Na\(^+\)-free medium was barely detectable. This and the evidence in 2 above indicates that the exchange carrier cannot catalyze net methionine uptake.

4. Cellular ATP and extracellular Na\(^+\), rather than transmembrane Na\(^+\), K\(^+\) or Na\(^+\) plus K\(^+\) gradients, are required for optimal amino acid accumulation. Cellular ATP and
extracellular Na\(^+\) both decrease the $K_m$ values for amino acid influx.

5. Cation gradient-induced amino acid movement in ATP-depleted cells was insufficient to account for accumulation under more physiological conditions.

6. The Na\(^+\) gradient hypothesis is not generally applicable to amino acid transport in these cells.
MONOVALENT CATIONS, TRANSPORT AND EXCHANGE DIFFUSION OF
NEUTRAL AMINO ACIDS IN EHRlich ASCITES CELLS

by

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A thesis submitted to the Faculty of Graduate Studies and
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for the degree of Doctor of Philosophy.

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ACKNOWLEDGEMENTS ............................................................... iii
LIST OF ABBREVIATIONS ..................................................... viii
LIST OF TABLES AND FIGURES ........................................... ix

CHAPTER 1. INTRODUCTION .................................................. 1
1.1. Historical Survey ....................................................... 2
1.2. General Characteristics of Amino Acid Transport ............. 5
   A. Carrier Mediation ................................................... 5
   B. Kinetic Considerations ............................................. 13
      (i). The Time-Course of Uptake ................................. 13
      (ii). The Effect of Substrate Concentration ................. 14
      (iii). Exchange Diffusion ......................................... 15
      (iv). The Effects of Temperature .............................. 15
      (v). The Effects of pH ........................................... 17
   C. Structural Requirements .......................................... 17
   D. The Role of Na\(^+\) and K\(^+\) ................................. 19
      (i). Net Transport and Na\(^+\) ................................. 19
      (ii). Net Transport and K\(^+\) ................................. 21
      (iii). Exchange Diffusion ....................................... 22
      (iv). Models of Na\(^+\) sensitive Transport Systems .... 22
E. The Requirement for Energy ........................................ 23
   (i). Energy Sources ........................................... 23
   (ii). Reaction-coupled Versus Flux-coupled Uphill Amino Acid Transport .......... 24
   (iii). Equilibration and Exchange Diffusion .... 27
   (iv). Influx and Efflux.................................... 28

1.3. Amino Acid Transport Systems in Various Tissues ..... 28
    A. Identification of Different Systems ......................... 28
    B. The Ehrlich Ascites Cell ..................................... 30
    C. Red Blood Cells ........................................ 32
    D. Brain ...................................................... 33
    E. Kidney ........................................................ 34
    F. Intestine .................................................. 35

1.4. Objectives of the Present Work ............................... 35

CHAPTER 2. MATERIALS AND METHODS ..................................... 39

2.1. Reagents ..................................................... 39
2.2. Maintenance and Preparation of Ehrlich Ascites Cells ........................................... 40
2.3. General Preincubation and Incubation Procedures ....... 40
2.4. The Measurement of Cellular Radioactivity ................. 42
2.5. The Calculation of $^{14}$C-Amino Acid Uptake .................... 43
2.6. The Measurement of Exchange Diffusion ......................... 45
2.7. Measurement of the Endogenous Free Amino Acid Pool .................... 46
2.8. The Measurement of Na\(^+\) and K\(^+\) ......................... 46
2.9. The Measurement of ATP ........................................ 47

CHAPTER 3. THE HOMOEXCHANGE DIFFUSION OF L-METHIONINE ..... 49
3.1. Theoretical ...................................................... 49
3.2. The Effect of Temperature ...................................... 51
3.3. The Effect of Na\(^+\) and K\(^+\) ................................. 54
3.4. The Effect of Cellular ATP ................................. 60
3.5. Summary ...................................................... 62

CHAPTER 4. THE UPTAKE OF METHIONINE IN THE ABSENCE AND
PRESENCE OF EXTRACELLULAR Na\(^+\) ................................. 64
4.1. Theoretical ...................................................... 64
4.2. The Endogenous Free Amino Acid Pool ...................... 64
4.3. The Effect of Other Neutral Amino Acids .................. 70
4.4. The Effect of Extracellular Methionine Concentration ... 75
4.5. The Effect of ATP Depletion ................................. 78
4.6. Summary ...................................................... 80

CHAPTER 5. NEUTRAL AMINO ACID UPTAKE, ATP AND MONOVALENT
CATION GRADIENTS .................................................. 81
5.1. Theoretical ...................................................... 81
5.2. On the Necessity of the Na\(^+\) Gradient ...................... 82
5.3. Movement of Monovalent Cations and Amino Acids in
ATP-Depleted Cells ................................................. 92
5.4. Amino Acid Movement in ATP-Containing and ATP-
Depleted Cells ...................................................... 108
5.5. The Effect of Na⁺ and ATP on Net Amino Acid Uptake ............................................. 111
5.6. Summary ................................................................. 118

CHAPTER 6. DISCUSSION ......................................................... 120
6.1. Net Amino Acid Transport ........................................... 120
6.2. Exchange Diffusion and Net Transport ......................... 130

CLAIMS TO ORIGINAL RESEARCH ................................. 137

BIBLIOGRAPHY ................................................................. 140
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-AIB</td>
<td>α-aminoisobutyric acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>DNP</td>
<td>2,4-dinitrophenol</td>
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<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<tr>
<td>K⁺</td>
<td>potassium (ions)</td>
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<tr>
<td>Na⁺</td>
<td>sodium (ions)</td>
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<tr>
<td>TPN⁺</td>
<td>triphosphopyridine nucleotide</td>
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<td>TCA</td>
<td>trichloroacetic acid</td>
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</table>
# LIST OF TABLES AND FIGURES

## TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>44</td>
</tr>
<tr>
<td>3-1</td>
<td>58</td>
</tr>
<tr>
<td>3-2</td>
<td>59</td>
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<tr>
<td>4-1</td>
<td>66</td>
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<td>4-2</td>
<td>67</td>
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<td>77</td>
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<td>79</td>
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<td>5-1</td>
<td>84</td>
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<td>107</td>
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<td>110</td>
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<td>5-12</td>
<td>116</td>
</tr>
<tr>
<td>5-13</td>
<td>117</td>
</tr>
</tbody>
</table>

## FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1</td>
<td>52</td>
</tr>
<tr>
<td>3-2</td>
<td>53</td>
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<td>55</td>
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<td>56</td>
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<td>3-5</td>
<td>61</td>
</tr>
<tr>
<td>4-1A</td>
<td>71</td>
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<tr>
<td>4-1B</td>
<td>71</td>
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<td>4-2A</td>
<td>72</td>
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<td>4-2B</td>
<td>72</td>
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<td>4-3</td>
<td>76</td>
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<td>86</td>
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<td>5-6</td>
<td>112</td>
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<td>5-7</td>
<td>114</td>
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<td>5-8</td>
<td>115</td>
</tr>
</tbody>
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CHAPTER 1

INTRODUCTION

Since Chambers (1) first demonstrated the physical existence of the plasma membrane with microdissection techniques, that structure and its associated functions have become the subjects of an ever increasing number of investigations. Consequently, the early concepts of the membrane as a static barrier which, (a), exhibits selective permeability towards diffusing ions and molecules strictly on the basis of their size, charge, and lipid solubility and (b), prevents the loss of essential cellular constituents, have been modified. The membrane is currently regarded as a dynamic component of the cellular mechanics which is capable of exerting control over the intracellular environment. Perhaps the major force behind this change in attitude was the repeated demonstration of the mediated transport of ions, sugars, and amino acids through the membrane. For instance, in 1960, Christensen (2) outlined the role of active transport in membrane function as follows; "These transport processes determine what solutes shall enter the cell and at what rates. They establish the internal environment of the cell. They permit the cell to gain metabolites from very dilute environments, and accordingly such processes
may be highly developed in simple organisms. In the higher organism, these processes determine which metabolites are exchanged among cells and which are not. They also permit the formation and control of a portable extracellular environment that has given the higher animal mobility and relative freedom from external conditions." The following sections contain a brief introduction to the subject of amino acid transport. The scope of the discussion will be limited to the aforementioned topic and no attempt will be made to treat the transport of sugars or ions.

1.1 HISTORICAL SURVEY

The earliest studies in the field of amino acid transport were performed in vivo. They demonstrated that when an animal received an amino acid by feeding, infusion or injection, most of the body tissues would accumulate the amino acid to a concentration greater than that in the blood or extracellular fluid. Van Slyke and Meyer (3) first observed this phenomenon in the dog while tracing the fate of infused casein hydrolysates. Further demonstrations of the concentration of amino acids by various tissues were reported over the next fifty years (4-22). It was apparent even to Van Slyke and Meyer that when amino acids move from a medium wherein they are dilute to one wherein they are more concentrated, a mechanism other than mere osmosis is involved.
Related investigations indicated that some amino acids compete with one another for "... the means by which cells concentrate amino acids presented to them ..." *(4,6,21,23-25).* For instance, Christensen et al (23) reported that "... amino acids which produced high concentrations in the organism when fed (proline, histidine, glycine, methionine, threonine, serine, valine and alanine) produced a diminution in the distribution ratio for glycine. Similarly when high levels of glycine were produced by feeding glycine, the distribution ratios for the other amino acids were reduced." The concentration and competition phenomena were also observed in kidney where, (a), structurally similar amino acids may be reabsorbed at different rates (26,27), (b), the rate of individual amino acid reabsorption approaches a maximum value (28-32) and (c), some amino acids compete with one another for reabsorption (28,29,32). Competition for transport between various amino acids has also been observed in intestine (33-38), pancreas (39,40), brain (41-44), bacteria (45,46), etc.

Competitive inhibition of transport among amino acids has been utilized in the characterization of specific transport systems. From the absence or presence of such inhibitions and the values of the relevant kinetic parameters, it is usually decided whether substrates are transported via the same or different pathways (39,40,47,48). In a comparison of the transport systems for amino acids in brain, intestine, kidney and

* Ref. 23
tumor, Neame (49) has described separate systems for the transport of acidic, basic, and neutral amino acids. However, there are various degrees of subgrouping within and overlap between the separate systems, particularly in brain and tumor.

Exchange diffusion is another phenomenon associated with the transport process (50-52). The first demonstration of transmembrane exchange diffusion of amino acids was reported by Heinz and co-workers (53-55) who observed that the rate of glycine exchange diffusion was approximately five times that of net transport (55). Furthermore, glycine influx due to exchange diffusion was apparently proportional to the cellular glycine level (56). A few years later, Wilbrandt and Rosenberg (50) wrote that such counter-transport phenomena "... should be expected in all systems in which the binding site (carrier) is mobile, but not in systems involving fixed binding sites ...". Furthermore, demonstration of such counter-transport phenomena between two transport substrates "... may be considered as proof that the two substrates share affinity for the carrier". Exchange diffusion between many amino acids has been demonstrated in a variety of tissues (47,48,57-74).

More recently, investigations have been directed towards determining the ionic and energetic factors which regulate amino acid transport systems. Thus it has been found that the presence of extracellular Na⁺ and K⁺ is necessary for optimal uphill transport of amino acids (45,57,75-92).
Metabolic energy is also required for the uphill transport of amino acids as conditions which lead to a reduction in the availability of metabolic energy or cellular ATP also inhibit uphill amino acid transport (45, 55, 71, 75, 84, 93-98). However, there is, at present, considerable controversy concerning the immediate energy source for uphill amino acid transport. Several investigations have indicated that the normal Na\(^+\) electrochemical or chemical potential gradient directly provides the required energy for uphill amino acid transport (90, 99-108) while others have shown that uphill amino acid transport cannot be correlated to the Na\(^+\) electrochemical or chemical potential gradient (89, 109) and so imply the existence of a direct link between available metabolic energy and uphill amino acid transport.

1.2 GENERAL CHARACTERISTICS OF AMINO ACID TRANSPORT

A. CARRIER MEDIATION

Osterhout (110, 111) and Lundegardh (112) were probably the first to suggest that transmembrane transport may involve transient binding of the transported substance to some membrane component. It is now generally accepted that membrane constituents called "carriers" participate in the transport of a variety of substances. The four essential elements of the carrier mechanism are as follows: (a), the combination -- at one surface of the membrane -- of the transport substrate with the
carrier to form a complex, (b), the movement of the complex from that surface of the membrane to the other, (c), the release of the transport substrate and (d), the return of the carrier to the first surface of the membrane. A mechanism such as that above would obviously be characterized by saturation kinetics, structural specificity for substrates and competition among substrates for transport (50,113). Furthermore, it is symmetrical and therefore capable of mediating the equilibration of the substrate across the membrane. Uphill transport, however, requires the introduction of an asymmetry by the "... coupled flow of another thermodynamic quantity from higher to lower thermodynamic potential" (114,115).

The basic features of the carrier mechanism have been incorporated into many schematic transport models, each of which supposedly explains the relevant experimental observations and predicts the capabilities of the transport system under various conditions. Several general kinetic models are most pertinent to the work reported herein and will therefore be discussed. To begin, there are three basic models which describe carrier mediated transport; one in terms of facilitated diffusion (carrier mediated transport which is independent of metabolic energy and at the steady-state, leads to equilibration, rather than accumulation, of the transport substrate) and the other two in terms of uphill, accumulative transport which is dependent upon metabolic energy.
FACILITATED DIFFUSION: MODEL 1. In the symmetrical system, if the free carrier and the carrier-substrate complex cross the membrane at equal rates, the net transport of substance S can be described as the sum of saturable influx and efflux processes. The net transport rate will be given by

\[ v = V_{\text{max}} \left[ \frac{S_1}{S_1 + K_m} - \frac{S_2}{S_2 + K_m} \right] \] \hspace{1cm} (1-1)

where \( v \) is the net transport rate, \( V_{\text{max}} \) is the maximum net transport rate, \( S_1 \) and \( S_2 \) are the extracellular and cellular concentrations of transport substrate S respectively and \( K_m \) is the Michaelis constant (50,116). The initial transport rate or influx (when \( S_2 = 0 \)) will be given by

\[ v = V_{\text{max}} \cdot \left[ \frac{S_1}{S_1 + K_m} \right] \] \hspace{1cm} (1-2)

or by the double reciprocal equation of Lineweaver and Burk (117);

\[ \frac{1}{v} = \frac{K_m}{V_{\text{max}}} \cdot \frac{1}{S_1} + \frac{1}{V_{\text{max}}} \] \hspace{1cm} (1-3)

Similar equations can be written to describe the efflux of \( S_2 \) (when \( S_1 = 0 \)). The system described above will exhibit saturation kinetics, structural specificity for substrates and competition among substrates and analogues.

Without altering any characteristics of the above system and making use of the tendency of substrates to compete for
transport, it is possible to observe transient uphill transport by a process known as counter-transport (50). The process itself is defined as the uphill transport of a substrate, S, in the direction of a large and preferably saturating concentration of a second substrate, R, which has been placed on one side of the membrane. The mechanism may be visualized as follows: S is first allowed to equilibrate between cellular and extracellular phases. R can then be added to the extracellular medium at a concentration larger than its $K_m$ value for transport. R will then interfere with the binding of S to the carrier at the external surface of the membrane thus inhibiting the unidirectional influx of S. The asymmetry which has been introduced becomes apparent when it is considered that the unidirectional efflux of S will remain unaffected until large amounts of R enter the cell. Until then, an inward gradient of R will exist and S will move out of the cell against its own gradient. The movements of S and R need not be equivalent unless S was initially present on both sides of the membrane at concentrations sufficient to saturate the carrier.

If the translocation of free carrier is assumed to be the rate-limiting step in the symmetrical system, the characteristics of the aforementioned processes would remain unchanged except that the net transport rate may be somewhat reduced due to the slowed rate at which the empty carrier returns to the external surface of the membrane. If $r$ is the ratio of the rate of travel of the complexed to that of the free carrier
and \( r \) is greater than 1, some function of \( r \) usually modifies both the \( K_m \) and \( V_{\text{max}} \) terms of equations (1-1) to (1-3) (116). The symmetrical system under these circumstances can mediate exchange diffusion, a capability which it lacks when \( r \) is equal to 1 (116). Exchange diffusion is defined as the increased rate of unidirectional flux of \( S \) due to the presence of \( S \) or \( R \) on the side of the membrane towards which the flux of \( S \) is directed. As this definition bears some superficial resemblance to that for counter-transport, the differences between the two processes should be stressed; exchange diffusion involves an increased rate of flux either uphill or downhill while counter-transport is concerned with an uphill flux the rate of which need not be increased.

The mechanism of exchange diffusion may be visualized as follows. Extracellular \( S \) is transported into the cell. The carrier must then return to the outer surface of the membrane in order to transport more \( S \) inward. As the mobility of the carrier-substrate complex is greater than that of the free carrier \((r > 1)\), the presence of \( S \) or \( R \) inside the cell will accelerate the return of carrier to the outer surface of the membrane, thus making more carrier available at that surface. The inward transport of extracellular \( S \) is therefore increased in rate. It should also be possible to demonstrate a stimulated efflux rate of \( S \) due to the presence of \( S \) or \( R \) outside the cell. Furthermore, the exchange of cellular and extracellular \( S \) (or \( R \)) is equimolar so that there is no net change in the concentra-
tion of S or S plus R on either side of the membrane.

UPHILL TRANSPORT: MODEL 2. The theoretical transformation of the facilitated diffusion system above into a carrier pump capable of mediating sustained uphill transport involves the imposition of an asymmetry on the carrier by metabolic means. The result would be the interconversion of the carrier between two forms, C and Z, (115) differing in their affinities for the transport substrate. Net uphill transport into the cell will be observed if, (a), C has the greater affinity for the substrate and (b), Z and complexes thereof are converted to C and the equivalent C-complexes on side 1 (extracellular surface) of the membrane while C and complexes thereof are converted to Z and the equivalent Z-complexes on side 2 (cellular surface) of the membrane. In order to write a rate equation for the net transport of S, the relative concentrations of both carrier forms on each side of the membrane and the respective affinities of both carrier forms for the transport substrate must be considered. In the simplest case, equation (1-1) will be applicable except that the $K_m$ term must be modified to include the aforementioned considerations. Thus, the rate equation again takes the form

$$v = v_{max} \left[ \frac{S_1}{S_1 + K_1} - \frac{S_2}{S_2 + K_2} \right] \quad \ldots \ldots \ldots \ldots \ldots (1-4)$$

where $K_1$ and $K_2$ are the modified $K_m$ terms. Furthermore, due to
the asymmetric nature of the system, $K_1$ is not equal to $K_2$. As the free and complexed forms of both $C$ and $Z$ do not necessarily travel through the membrane at the same rate, some function of $r_C$ and $r_Z$ (the ratio of the rate of travel of the complexed to the free forms of $C$ and $Z$) may modify the $V_{\text{max}}$, $K_1$ and $K_2$ terms of equation (1-4). Like the symmetrical system, this system will exhibit saturation kinetics, structural specificity for substrates, competition among substrates and analogues and counter-transport (115). If the asymmetry of the system is not great and both $r_C$ and $r_Z$ are each greater than 1, the system will mediate exchange diffusion. In the presence of metabolic inhibitors, the interconversions of $C$ and $Z$ are blocked, $K_1$ becomes equal to $K_2$ and accumulation ceases. The system still theoretically and qualitatively resembles Model 1.

UPHILL TRANSPORT: MODEL 3. This proposal was forwarded by Heinz and Walsh (56) and can be considered as a variant of Model 2. In this system, $C$ combines with $S$ at the outer surface of the membrane. The $CS$ complex then translocates to the inner surface where it must first dissociate before $C$ is catalytically and immediately converted to $Z$. The presence of cellular $S$ would thus tend to protect the $C$ forms from immediate deactivation. $Z$, which cannot combine with $S$, returns to the outer surface of the membrane where it is converted to $C$ by an irreversible, energy requiring and rate-limiting process. Furthermore,
there is a provision for the unmediated diffusion of S through (functional if not physical) pores in the membrane. The present system will exhibit saturation kinetics, structural specificity for substrates, competition among substrates and analogues, counter-transport and exchange diffusion. Exchange diffusion in this system can be mediated only by the C form of the carrier and is therefore dependent on the presence of that form. In the presence of metabolic inhibitors, the conversion of Z to C is blocked and accumulation ceases. Unlike Models 1 and 2, the present system would lose its ability to mediate exchange diffusion and counter-transport with increasing time in the presence of metabolic inhibitors as all the carrier would be gradually converted to the Z form.

That which remains to be discussed is the nature of, and the forces responsible for the C-Z interconversions. It has been suggested (103) that the carrier must bind Na⁺ in order to become activated (C form). The loss of the Na⁺ either by dissociation or through displacement by K⁺ would constitute the deactivation of the carrier (Z form). The C-Z type of asymmetry which results in net uphill transport of substrate into the cell would therefore be directly dependent on the presence of a Na⁺-K⁺ asymmetry across the cell membrane such that, (a), the extracellular concentration of Na⁺ exceeds that of the cell and (b), the cellular concentration of K⁺ exceeds that of the extracellular fluid. The maintenance of the asymmetric Na⁺-K⁺
distribution would be dependent upon metabolic energy in the form of ATP.

A second possibility is that metabolic energy in the form of ATP is itself responsible for the C-Z type of interconversions. The role of Na⁺ and K⁺ in such a system would be to augment the effect of the C-Z interconversions by participating in the transport mechanism in the manner outlined above. These two mechanisms will be discussed further in section 1.2.E.

B. KINETIC CONSIDERATIONS

(i) The Time-Course of Uptake

Experimentation has repeatedly verified the fact that amino acids which are transported into cells and tissues reach a steady-state level therein (41, 63, 65, 76, 93, 97, 118-126). Heinz (53) demonstrated that the time required to reach half the steady-state level for glycine uptake by Ehrlich ascites cells was constant over a 10-fold range of extracellular glycine concentration. Glycine uptake was therefore first order with respect to time and followed the equation

\[
\frac{S_2}{S_1} = \frac{S_2}{S_1} \cdot \left[ 1 - e^{-kt} \right]_{t=\infty} \quad \text{............(1-5)}
\]

where \( t \) is the time and \( k \) is a rate constant. The expression
above is important in that it can relate the magnitudes of the initial rate and the steady-state level of uptake.

(ii) The Effect of Substrate Concentration

The first two models in section 1.2.A. describe the net transport of S in terms of saturable influx and efflux processes. However, Heinz has attributed the net transport of glycine into Ehrlich ascites cells to a "pump and leak" system; while the saturable process of uphill transport pumps glycine into the cell (53), the amino acid escapes from the cell by the process of passive diffusion the rate of which is directly proportional to the concentration gradient of glycine across the cell membrane (56). The rate equation for the net transport of S then becomes

\[ v = V_{max} \left[ \frac{S_1}{S_1 + K_1} \right] - k_2 (S_2 - S_1) \]

where \( k \) is the rate constant for the efflux process and subscripts 1 and 2 again refer to parameters associated with the extracellular and cellular compartments respectively. Wilbrandt and Rosenberg (50), however, have stressed that "... linear kinetics may well be observed in a carrier system if the saturation is low". And indeed, Christensen and Handlogten (127) have reported that the exit processes of several amino acids exhibit high \( K_m \) values. Thus although the efflux may be carrier
mediated, the asymmetry of the system can be great enough to cause the efflux to appear nonsaturable.

(iii) Exchange Diffusion

Since Heinz first demonstrated the homoexchange diffusion (exchange of S) of glycine in Ehrlich ascites cells (53, 54), subsequent work has shown that the rate of glycine influx due to exchange diffusion was, (a), five times that of net transport (55) and (b), roughly proportional to the cellular glycine level (56). Paine and Heinz (128) found that compounds which did not inhibit net glycine uptake did not usually participate in heteroexchange diffusion (exchange of S and R) with glycine. Oxender and Christensen (47, 48) extended the type of study begun by Paine and Heinz. They observed that the influx of those amino acids which participated in exchange and heteroexchange diffusion exhibited an increased $V_{\text{max}}$ when the cells were preloaded with exchangeable amino acid. This result is consistent with the suggestion that during exchange diffusion, more carrier is available at one surface of the membrane than during net transport. Heteroexchange and exchange diffusion of amino acids has also been examined in brain (45, 70, 129, 130), intestine (67-69), kidney (72, 73), pancreas (40, 74), etc.

(iv) The Effects of Temperature

Both net transport and exchange diffusion of amino acids
are temperature dependent in that an increase in temperature results in a rise of the rates of net transport and exchange diffusion \((48,53,60,65,73,75,109,120,122,131-134)\) so long as the temperatures in question remain above \(0^\circ\) and below \(40^\circ\)C. This dependence on temperature is consistent with other evidence which indicates the chemical nature of the transport process. It was found that this temperature coefficient or \(Q_{10}\) for the uphill transport of most amino acids lay in the range between 1.7 and 3.5 when estimated between \(25^\circ\)C and \(37^\circ\)C. The uptake of L-tyrosine into rat diaphragm is an exception, the \(Q_{10}\) for that process being only 1.4 \((135)\). Stein \((136)\) has compared the temperature coefficients and activation energies of passive cation permeability of various cell membranes while Jacquez et al \((132)\) have done the same for the mediated transport of several amino acids. The activation energies (Arrhenius \(E\) values) for amino acid transport usually lay in the range between 10 and 30 kcal/mole while those for passive amino acid, \(\text{Na}^+\) and \(\text{K}^+\) flux were distributed between 2 and 20 kcal/mole. It is interesting to note that the activation energy for free \(\text{Na}^+\) diffusion in water is approximately 5 kcal/mole while that for its diffusion into highly cross-linked cation exchange resins was approximately 9 kcal/mole. Comparison of the above \(E\) values suggests that the mechanism which transports amino acids across the cell membrane does not do so through water-filled pores.
(v) The Effects of pH

Christensen and co-workers have demonstrated that the transport of amino acids into Ehrlich ascites cells is influenced by the extracellular pH, the uptake and efflux of several amino acids exhibiting optimum activity at pH 7.4 (48). Furthermore, the separate transport systems of the Ehrlich cell showed different patterns of pH sensitivity (48,138). From measurements of glycine uptake into Ehrlich ascites cells as a function of extracellular pH and glycine concentration, Heinz (78) has concluded that protons do not compete with the transport substrates for the same site on the carrier. Heinz et al (137) also found that unlike the transport of most other amino acids into Ehrlich cells, glutamate uptake was stimulated by a condition of mildly acidic pH. The stimulation is believed to be associated with the protonation of the carrier rather than the substrate.

C. STRUCTURAL REQUIREMENTS

When the typical monoamino-monocarboxylic acid structure, R-CH(NH$_2$)-COOH, is considered the standard to which other related compounds are compared, the structural specification required for the uphill transport of such compounds appear quite broad. For example, removal of the carboxyl group results in a series of primary amines which are transported by Ehrlich ascites cells (138) and mast cells (139,140) but not intestine (141). Ehrlich cells will also transport taurine (142) but not
the corresponding α-amino derivative (2). However, removal of
the amino group results in a series of aliphatic acids which
are not known to be accumulated (143). If the amino group is
merely displaced from the α to the β position, the resulting
compound can still be concentrated (2,144,145). Methylation of
the α-amino group reduces the rate of transport but the methy-
lated amino acids are concentrated in a number of tissues (49,
146-148). The presence of the α-hydrogen is not essential for
transport as proline, hydroxyproline, α-aminoisobutyric acid
and cycloleucine are all well accumulated (145,149).

The α-amino acids differ from one another in the composi-
tion of their side chains. These differences are obviously
the basis for the substrate specificities exhibited by many
amino acid transport systems (section 1.3). Therefore side-
chain modification in relation to transport will not be dis-
cussed here beyond the following few comments. α-Amino acids
with long and/or branched aliphatic side chains are not usu-
ally well accumulated while their shorter analogues are easily
concentrated (2,58). Furthermore, introduction of halogens
into the side chain reduces the uphill transport of the com-
 pound (150). However, α-amino acids with additional carboxyl
or amino groups on the side chain are accumulated in Ehrlich
ascites cells (131,137,151) as are those with aromatic groups
(48,63,64,131).

From a number of investigations concerned with the trans-
port of D and L amino acid forms, it has been observed that
both isomers "... may be transported although generally not at the same rate or to the same extent" (84). For instance, the uptake of L-amino acid isomers in brain and intestine is usually faster and more extensive than that of the corresponding D form (21,95,118,152-158). In Ehrlich ascites cells, however, the transport of the L isomer may (137,152,159,160) or may not (57,63) surpass that of the D form. Thus in commenting on the significance of steriospecificity in the amino acid transport systems of the Ehrlich ascites cell, Johnstone and Scholefield (84) wrote "Other structural features .... appear to play a much more important role in determining the transportability of an amino acid".

D. THE ROLE OF Na⁺ AND K⁺

(i) Net Transport and Na⁺

The first indication that the process of net amino acid transport was sensitive to the monovalent cation composition of the extracellular medium appeared in 1952 when Christensen et al (57) reported that partial replacement of extracellular Na⁺ by either choline⁺ or K⁺ reduced the accumulation of glycine by Ehrlich ascites cells. The net transport of many amino acids (α-aminoisobutyric acid, alanine, glutamic acid, glycine, histidine, lysine, methionine, proline, serine, tyrosine, etc.) in various tissues has since been shown to be sensitive to extracellular Na⁺ (76,79-83,86-90,91,100,101,105,107,109,161-173).
The net uptake of all amino acids may not necessarily be $\text{Na}^+$-dependent; that of arginine, cycloleucine, leucine, lysine, methionine, phenylalanine and valine in various tissues does not exhibit a sensitivity to $\text{Na}^+$ (91,146,164-166). However the possibility that this $\text{Na}^+$-insensitive uptake is not due to net transport but to exchange diffusion with endogenous amino acids, has not been eliminated.

The process of net transport -- as described in Models 1 and 2 of section 1.2.A. -- is the sum of separate influx and efflux processes. It has become apparent that when the transport is $\text{Na}^+$-dependent, the unidirectional influx of amino acids is the process sensitive to the level of extracellular $\text{Na}^+$ (81, 88,90,105,109,161,163,164,167,169) while the unidirectional efflux is, in some cases, sensitive to the cellular $\text{Na}^+$ level (81,107,169). The presence of increasing concentrations of extracellular $\text{Na}^+$ is known to result in a decreased Michaelis constant (81,88,90,109,169) or a decreased Michaelis constant as well as an increased maximum rate of amino acid influx (105, 161,163,164,167). Similarly, the Michaelis constant for amino acid efflux is decreased in the presence of high cellular $\text{Na}^+$ levels (107,169). $\text{Na}^+$ is apparently essential for the optimal operation of the carrier mediated net transport of many amino acids.

The $\text{Na}^+$-sensitive net accumulation of several amino acids has been shown to result in an increased cellular $\text{Na}^+$ level
Furthermore, Shultz and Curran (90) have demonstrated that the Na\(^+\)-sensitive influx of alanine into rabbit ileum causes a proportional increase in Na\(^+\) influx. Shafer and Jacquez (176) have observed that the Na\(^+\)-sensitive uptake of α-aminoisobutyric acid into Ehrlich ascites cells is accompanied by an equimolar influx of Na\(^+\) while the Na\(^+\)-insensitive uptake of phenylalanine was not accompanied by any detectable Na\(^+\) influx. Na\(^+\) is apparently co-transported into the cell along with amino acids. The role of Na\(^+\) in Na\(^+\)-sensitive amino acid transport therefore appears to be that of an essential co-substrate.

**(ii) Net Transport and K\(^+\)**

The role of K\(^+\) in amino acid transport is not as well defined as that of Na\(^+\). Extracellular K\(^+\) concentrations of up to 10 mM are apparently necessary for the optimal operation of uphill amino acid transport (57,75,78,85,92,94,177). Higher concentrations may (47,105,109,145,177) or may not (78,79) adversely affect amino acid accumulation. It has been suggested that extracellular K\(^+\), when present at concentrations in excess of 10 mM, acts as a competitive inhibitor of Na\(^+\) in Na\(^+\)-sensitive transport systems (103,177). It is interesting to note that respiration is stimulated by extracellular K\(^+\) concentrations of up to 10 mM (178,179) but inhibited at higher extracellular K\(^+\) concentrations (178). Although cellular K\(^+\) levels have no effect on amino acid influx (57,78,79), a net loss of cellular K\(^+\) has
been repeatedly associated with the net accumulation of several neutral and basic amino acids \((93, 99, 131, 145, 174, 175, 180, 181)\). Furthermore, Hempling and Hare \((182)\) have observed that both the influx and efflux of \(K^+\) were increased during the accumulation of glycine by Ehrlich ascites cells.

(iii) Exchange Diffusion

Compared to net uphill transport, the cation requirements for amino acid exchange diffusion have received little attention. However, it has been shown that in Ehrlich ascites cells the exchange diffusion of methionine \((92)\) and phenylalanine \((132)\) as well as the heteroexchange diffusion between methionine and cycloleucine \((183)\) do not require the presence of extracellular \(Na^+\). As the exchange (and heteroexchange) diffusion of methionine is a very rapid process, it was largely complete within the first few minutes of incubation \((92)\). Therefore little time was available to alter the cellular ionic composition the effects of which remain unassessed.

In pigeon red cells and rabbit reticulocytes, the exchange diffusion of alanine \((66, 91, 164)\), glycine and valine \((91)\) as well as the heteroexchange diffusion between these three amino acids \((91, 164)\) is \(Na^+\)-dependent.

(iv) Models of \(Na^+\)-Sensitive Transport Systems

The models of facilitated diffusion and uphill transport systems described in section 1.2.A. can be expanded to include \(Na^+\) as an essential co-substrate in the transport
of $S$. In such models, $Na^+$ would combine with a specific site on the carrier separate from the amino acid binding site (103, 108, 176).

Models 2 and 3 for uphill transport have been previously treated as simple expansions of Model 1 (the facilitated diffusion system) the carrier of which, when combined with $Na^+$, becomes the C form. However, further comment is required concerning the case in which the C-Z interconversions do not involve $Na^+$ binding but $Na^+$ acts as a co-substrate which, when bound to C, augments the asymmetry inherent in the C-Z system.

If, in Model 2, the inherent asymmetry between C and Z is not great, the transmembrane distribution of $Na^+$ (and probably $K^+$) may contribute to the net transport rate. In the event of a large asymmetry between C and Z, the transmembrane $Na^+$ (or $K^+$) gradient would make only a limited contribution to the net transport rate. Extracellular $Na^+$, however, would be expected to influence that rate. It is interesting to note that Model 3 is so constructed that if $Na^+$ is a co-substrate, then the transmembrane distribution of $Na^+$ (and probably $K^+$) can effect the net transport rate even though the C-Z asymmetry is very large ($Z$ cannot combine with substrates).

E. THE REQUIREMENT FOR ENERGY

(i) Energy Sources

In thermodynamic terms, the "uphill" flow of a substance
is a process which requires energy or a driving force. The thermodynamic driving force responsible for the uphill flow in question -- uphill transport of amino acids -- must itself result from the flow of some component of the system in question from higher to lower thermodynamic potential (114). There are three basic driving forces available to the carrier system mediating the transport of S (184,185); (a), the electrochemical potential gradient of the substrate itself, (b), the electrochemical or chemical potential gradient of solutes other than the substrate which can interact with the system and (c), the action of some chemical (metabolic) reaction. Since the transport substrates in question are essentially neutral (possessing no net charge) amino acids, source (a) could not bring about their accumulation but only their equilibration between cellular and extracellular compartments. Uphill transport driven by source (b), usually called "flux-coupled transport", includes counter-transport (60), heteroexchange diffusion and ion gradient-induced amino acid accumulation (81,100,102,106,107,169). Transport driven by source (c) is usually called "reaction-coupled transport". Although the uphill transport of amino acids (46,186) and sugars (187-190) into several microorganisms as well as that of amino acids into various animal tissues (45, 54,55,71,75,84,93-96,191,192) has been repeatedly associated with the availability of metabolic energy, several investigators have proposed that the driving force responsible for the
uphill transport of amino acids into animal tissues is the Na\(^+\) (and K\(^+\)) gradient(s) \((90,99,103-108,193)\). The apparent dependence on the availability of metabolic energy in the form of ATP would therefore be indirect and due to the direct dependence of the transport system maintaining the Na\(^+\) (and K\(^+\)) gradient on metabolic energy \((103,193)\).

(ii) Reaction-Coupled Versus Flux-Coupled Uphill Amino Acid Transport

The evidence in support of reaction-coupled amino acid transport consists mainly of the repeated demonstration that under conditions known to decrease the availability of metabolic energy the accumulation of amino acids by the tissue or bacterium in question ceases \((42,46,54,55,75,84,93-96,123,186,191,192)\). Furthermore, Abadom and Scholefield \((76,97)\) demonstrated that glycine uptake into rat brain cortex slices is a direct function of the cellular level of 7 minute acid labile nucleotide phosphate. Evidence has also been presented which suggests that only small amounts of ATP or similar compounds may be required to drive amino acid accumulation. For example, within 5 minutes of adding DNP to the extracellular medium, the incorporation of glycine into the proteins of Ehrlich ascites cells stops \((84,119)\) and the release of \(^{32}\)P from previously labelled ATP is practically complete \((194)\). However, the rate of glycine uptake remains unaffected for 5 additional minutes.
None of the above evidence is inconsistent with the claim that Na\(^+\) and K\(^+\) gradients are the primary driving forces for amino acid transport because the effects on the amino acid transport could have been due to changes in the Na\(^+\) and K\(^+\) distributions across the membrane.

The evidence in support of flux-coupled amino acid transport consists mainly of the demonstration that when metabolic energy is made unavailable, net amino acid movement can be correlated to the magnitude (100,106) and direction (100,107) of the transmembrane Na\(^+\) gradient. However, the magnitude of the Na\(^+\) gradient-induced accumulation is always much less than that observed when metabolic energy is available (106,107). This difference has been attributed either to the additional influence of the K\(^+\) gradient present in unpoisoned cells (106) or to the rapid dissipation of the ion gradients in question (107). Indeed it has been shown that when metabolic energy is available, the potential energy in the Na\(^+\) plus K\(^+\) gradients is of sufficient magnitude to drive amino acid accumulation (193). Yet the efficiency of the gradient-induced amino acid movement is still in question as a similar demonstration in the absence of metabolic energy is lacking. Two further observations are in apparent contradiction to the evidence supporting flux-coupled transport as they clearly indicate the participation of metabolic energy in amino acid accumulation. Respiring cells, but not cells which have been poisoned with cyanide, will accumulate
amino acids, (a), when there is no Na\(^+\) or K\(^+\) gradient across the cell membrane (106) and (b), when there is a reversed Na\(^+\) or Na\(^+\) and K\(^+\) gradient across the cell membrane (89,164,193).

(iii) Equilibration and Exchange Diffusion

In thermodynamic terms, only the electrochemical potential gradient of the transport substrate, S, need be considered as the energy source driving equilibration of S between cellular and extracellular compartments. However, such equilibration may proceed at a very slow rate. Since the equilibration of most amino acids proceeds at a fairly rapid rate, it therefore seems possible that a source of energy other than or in addition to the electrochemical potential gradient of S can drive the equilibration. Furthermore, the fact that the rate of carrier mediated downhill transport of Na\(^+\) into frog skin can be inhibited by cyanide (195) supports that possibility.

Because the process of exchange diffusion does not result in any net change in the amount or concentration of the transport substrate on either side of the membrane, no thermodynamic work need be associated with it. If, however, transport is mediated as described in Model 3, then exchange diffusion, which is catalyzed by the C form of the carrier, would be dependent on metabolic energy for the production of the C form. Although exchange diffusion is apparently unaffected when the availability of metabolic energy is made limiting (45,59-62,84,96,98),
the effect of allowing the carrier to become deactivated (Z form) by prolonged pretreatment with metabolic inhibitors has not been assessed.

(iv) Influx and Efflux

When metabolic energy is made unavailable, the influx of amino acids into Ehrlich ascites cells (54,55) and of sugars into yeast cells (190) is markedly reduced while the efflux processes of these substances remain unaffected (54,119,190). In E. coli, however, not only is the influx of galactosides inhibited (196), but their efflux is accelerated (187,188,197). Thus in Ehrlich ascites cells and yeast, the C to Z transformation may be independent of metabolic energy which is involved only in the Z to C transformation (115), whereas in E. coli, it appears as if the C to Z transformation requires metabolic energy while the Z to C conversion may not.

1.3 AMINO ACID TRANSPORT SYSTEMS IN VARIOUS TISSUES

A. IDENTIFICATION OF DIFFERENT TRANSPORT SYSTEMS

When two amino acids interact to inhibit one another's transport, the interaction is considered evidence that the amino acids in question have some degree of affinity for a common transport system or carrier. Conversely, in the absence of such inhibitive interaction, they are thought to be transported by separate systems. However, when one amino acid markedly inhibits
the uptake of a second amino acid while the second barely affects the transport of the first, the interpretation becomes more involved. Christensen (146) has summarized the various types of inhibitive interactions between amino acids and discussed their significance in terms of the identification of different transport systems. He has also illustrated how some of these interactions may be used in the identification of distinct systems.

When one amino acid inhibits the uptake of another, it could be acting either as a competitive inhibitor or a competitive substrate. Kinetic criteria have therefore been proposed to identify competitive substrates transported by a single system (149,198). If a component of the total uptake of two amino acids, A and B, is mediated by the same system, each amino acid should competitively inhibit that component so that the apparent $K_m$ and $K_i$ values for A are equal and the apparent $K_m$ and $K_i$ values for B are equal. Furthermore, a third amino acid should have the same apparent $K_i$ value when used to inhibit that component of uptake of either A or B.

The identification of a distinct system which mediates the transport of several amino acids, some or all of which are also transported to various degrees by other distinct systems, becomes a complex process and additional evidence to support the conclusions derived from inhibition studies is often necessary. Thus the sensitivity of the transport processes to temperature, pH and cations is frequently examined and the results used to
further characterize specific systems.

B. THE EHRLICH ASCITES CELL

Six distinct systems are thought to be responsible for amino acid transport in these cells; three of them transport neutral amino acids, one acidic, one basic and one $\beta$-amino acids. The substrate specificity is not of the highest order so that the uptake of a particular amino acid may be mediated predominantly by a given system yet one or more other systems may also contribute to its total uptake.

The neutral amino acids are apparently transported by the "alanine-preferring" (A), the "leucine-preferring" (L), and the "alanine-serine-cysteine" (ASC) systems (47,48,146,199). The A system will accept all neutral amino acids and small basic amino acids (138) but transports the smaller neutral amino acids with greatest efficiency (48,49,146). The larger and more branched neutral amino acids are transported predominantly by the L system (48,49,146,160,200). Some neutral amino acids, such as methionine (48,161) and cycloleucine (48), are transported equally well by both A and L systems. It has been shown that N-methylation of an amino acid abolishes all affinity for the L system (147) and probably the ASC system as well (146, 199). The preferred substrates of the ASC system are alanine, serine, cysteine and other neutral amino acids of intermediate chain length (146,199). The activity of both the A and the ASC
systems is dependent upon the presence of extracellular Na\(^+\) while that of the L system is not (48,146,161). Substrates predominantly transported by the A and ASC systems are well accumulated and do not readily participate in exchange and heteroexchange diffusion (47,48,146,199). Conversely, amino acids handled by the L system are not well accumulated but readily participate in exchange and heteroexchange diffusion (47,48,146). The A and ASC systems are therefore thought to mediate net uphill transport but little if any exchange diffusion while the L system is believed to catalyze exchange diffusion and some net uphill transport (48,146). In view of the large overlapping of substrate specificities, it is surprising that the limited accumulation of large and branched-chain neutral amino acids was attributed to the action of the predominantly exchanging L system and not to the cross reaction of those substrates with the A and ASC systems.

\(\beta\)-Alanine and taurine are transported predominantly by a \(\beta\)-system (144,146) which will also accept \(\alpha,\beta\)-diaminopropionic acid \(\alpha\)-aminobutyric acid (49,144). All the substrates above can apparently cross react with the A system (144). Like the A system, the \(\beta\)-system is Na\(^+\)-dependent and does not mediate exchange diffusion (146).

The transport of glutamic acid is apparently mediated by an acidic system (137) which has some affinity for neutral amino acids such as glycine, \(\alpha\)-aminoisobutyric acid, alanine and
methionine (49) but none for aspartic acid (137).

The "lysine-accepting" or Ly⁺ system mediates the transport of basic amino acids such as α,β-diaminopropanoic and butyric acids, ornithine, arginine, lysine and homoarginine (138, 151, 201). The Ly⁺ system can catalyze net uptake, exchange and heteroexchange diffusion (138, 201) and functions best in the absence of Na⁺ (138). This system also has some affinity for larger neutral amino acids, particularly in the presence of Na⁺, apparently because such substrates plus Na⁺ are analogous to a dibasic amino acid as far as the Ly⁺ carrier is concerned (201).

C. RED BLOOD CELLS

The systems described below are similar to those which have been defined for the Ehrlich ascites cells and have been identified in pigeon erythrocytes and rabbit reticulocytes.

The transport of the neutral amino acids is distributed among three systems: the "glycine", the "alanine-serine-cysteine-proline" (ASCP), and the L systems. The A system is absent in these cells just as the glycine system does not appear in Ehrlich ascites cells (146). The glycine system (66, 91, 146, 202) will accept glycine, proline, sarcosine and N-ethyl-glycine as substrates and is Na⁺-dependent (81, 163, 164). Exchange diffusion has not yet been demonstrated with this system (146) which appears to be dependent upon the normal inward Na⁺ gradient for the accumulation of substrates (102). In addition to its
preferred substrates, the ASCP system (66,91,146,199) will also handle some other neutral amino acids such as glycine, threonine and valine (66,91,199). This system mediates Na⁺-dependent net uptake, exchange and heteroexchange diffusion (91,146,164). The L system which handles the larger neutral amino acids, is independent of Na⁺ and does not catalyze exchange or heteroexchange diffusion in the pigeon red blood cell (91) but does mediate those processes in the rabbit reticulocyte and erythrocyte (66,164).

The β-system in these cells appears very similar to that in Ehrlich ascites (91,135).

The transport of basic amino acids in rabbit reticulocytes is mediated by a distinct system (203) which may require Na⁺. The uptake catalyzed by this system is not concentrative and does not require metabolic energy. Neutral amino acids, particularly in the presence of Na⁺, will interact with the basic system.

As the rabbit reticulocyte matures and becomes an erythrocyte, the cell loses the glycine, the ASCP, the basic and the β-amino acid systems (66,204). The L system, however, remains intact (66).

D. BRAIN

As many as seven systems have been reported to transport amino acids in brain. The separate systems have been identified
according to the substrates they handle and are referred to as the, (a), small neutral, (b), large neutral, (c), small basic, (d), large basic, (e), acidic and (f), the N-methylated amino acid systems as well as (g), the γ-aminobutyric acid system (70, 148, 205). The small neutral and both basic systems are known to be dependent upon Na⁺ for optimal activity while the large neutral and acidic systems are not (89). The acidic, both basic and both neutral systems are apparently capable of mediating exchange and heteroexchange diffusion (70). There is also considerable interaction between the separate systems (41, 43, 49, 148, 205-208).

**E. KIDNEY**

Amino acid transport in kidney is mediated by five systems: an acidic (49, 209, 210), a basic (31, 49, 124, 209-214) and a neutral amino acid system (31, 49, 124, 170, 209, 213, 214) as well as an imino acid system -- which also accepts glycine -- (49, 215) and a glycine system (146, 209, 213, 215, 216). The basic (170), neutral (83, 88) and glycine (83) systems are all Na⁺-sensitive. The basic system can mediate exchange and heteroexchange diffusion but only in the presence of Na⁺ (88). As in other tissues, the neutral amino acids are able to interact with practically all other systems (49, 209).
F. INTESTINE

The most striking feature concerning intestinal amino acid transport is the complete absence of a system capable of transporting acidic amino acids (49,153,157,217). However, there is a glycine system which accepts sarcosine, proline and hydroxyproline (49,69,218-221), a system which transports neutral amino acids (35,49,141,153,217,218) and a system for basic amino acids (49,153,218,219,222-225). Both the neutral (90,226,227) and the basic (90) systems are Na⁺-sensitive. As in other tissues, interactions between systems are quite common (49).

1.4 OBJECTIVES OF THE PRESENT WORK

The work to be described is, in general, concerned with the ionic and energy requirements of exchange diffusion and net uphill transport of neutral amino acids in Ehrlich ascites cells.

The exchange diffusion of neutral amino acids in these cells is mediated by the L system which is insensitive to extracellular Na⁺ (48). It is therefore not surprising that methionine exchange diffusion has apparently been shown to be independent of extracellular Na⁺ (92,183). However, the rapidity of this process at the temperatures used for its estimation (15 to 20°C) is considerable so that the exchange was largely complete after the first few minutes of incubation (92). Under such circumstances, the effects of extracellular monovalent cations may have been missed. Furthermore, the design of the experimentation
was such that the cellular ionic composition at the start of the exchange was always normal (low in Na\(^+\), high in K\(^+\)) and had little opportunity to undergo any but modest changes during the short incubation period. The effects of the cellular ionic composition have therefore remained unassessed. An examination of the effects of both cellular and extracellular monovalent cations on exchange diffusion at a temperature which would allow slower progress of the reaction was therefore of interest.

If net transport and exchange diffusion can be catalyzed by the same carrier, then at least one step in the net transport mechanism is rate-limiting. The models described in section 1.2.A. suggest two different rate-limiting steps in the net process which are bypassed during exchange diffusion: (a), the return of the free carrier to the outer surface of the membrane (Models 1 and 2) and (b), the energy dependent conversion of the Z form to the C form (Model 3). Although experimentation has shown that exchange diffusion is unaffected by metabolic inhibitors (45,59-62,84,96,98), these investigations were not designed to examine the prediction inherent in Model 3; that exchange diffusion, being dependent on the presence of the C form, could be affected by prolonged pretreatment with metabolic inhibitors which would block Z to C, but not C to Z conversions. If this prediction is not experimentally borne out, the rate-limiting step in the net process cannot be the Z to C
conversion (carrier activation) and the validity of Model 3 becomes questionable.

In addition to the incompletely defined ionic and energy requirements for the exchange process, the functional capabilities of the carrier mediating such exchange -- the L system -- are also unclear. Oxender and Christensen (48) have stated that the predominantly exchanging, Na⁺-insensitive L system is capable of mediating some net uphill transport. This widely accepted conclusion was based mainly on the evidence that substrates usually handled by the L system could undergo net accumulation. However, two other possible interpretations have not been ruled out experimentally; (a), the substrates in question undergo net accumulation because they cross react with another system capable of mediating net uphill transport and (b), the apparent net accumulation of the substrates in question is really an exchange process with endogenous amino acids. It is therefore of interest to, (a), assess the contribution of exchange diffusion to the total uptake of these amino acids and (b), determine whether or not the L system can mediate net transport.

Finally, turning to the subject of net uphill transport, there is presently considerable controversy concerning the immediate energy source for that process. Eddy (106) has shown that the Na⁺ gradient can drive a transient net uphill transport of amino acid in cyanide poisoned tumor cells which is approximately one third the uptake observed in respiring cells. The discrepancy
in activity was attributed to the additional contribution of the K⁺ gradient present in the respiring cells. Jacquez et al (193) have shown that in respiring Ehrlich ascites cells, there is indeed sufficient potential energy in the transmembrane distribution of Na⁺ plus K⁺ to drive the net accumulation of amino acid. However, as a demonstration of the gradient-induced transport in poisoned cells with suitable Na⁺ and K⁺ gradients was lacking, the efficiency of flux-coupled relative to reaction-coupled amino acid transport is still in question. It is therefore of interest to examine and compare the net uphill transport driven by the Na⁺ plus K⁺ gradient in both the presence and absence of available metabolic energy in the form of ATP.

The choice of L-methionine as the principal substrate with which these investigations would be performed was made on the basis of its ability to participate in both exchange diffusion and net uphill transport (48) and its reputed affinity for both the A and L systems of the Ehrlich ascites cell (48,161).
CHAPTER 2

MATERIALS AND METHODS

2.1 REAGENTS

(Carboxyl\(^{14}\)C\) inulin (1-3 mc/g), \((1-^{14}\)C\) glycine (2-10 mc/m mole), \(L-(Me-^{14}\)C\) methionine (10-15 mc/m mole), \(L-(1-^{14}\)C\) leucine (15-25 mc/m mole), and \({^{14}\)C\}) thiourea (10-20 mc/m mole) were purchased from the New England Nuclear Corporation of Boston, Massachusetts. Stock solutions, containing 0.1 mc/ml, were made with each of the above. Unlabeled amino acids and thiourea of the best available grade were purchased from the Nutritional Biochemicals Corporation of Cleveland, Ohio. Working solutions, aliquots of which were used in experiments, were prepared by diluting portions of the stock solutions and adding the appropriate unlabeled compound in amounts sufficient to produce a 60 mM solution. The specific activities of the working solutions were either 180-210, 360-420 or 720-840 dpm/nmole. The stock and working solutions were stored at \(-20^\circ\)C. Portions of these solutions were diluted and counted regularly to check for the decay of the radioactivity, but no decay could be detected.

Glycyl-glycine, TPN\(^+\), hexokinase (Sigma, Type III), and glucose-6-phosphate dehydrogenase (Sigma, Type XI) were purchased from Sigma Chemical Company of Saint Louis, Missouri. Disodium ATP was the product of the Nutritional Biochemicals
All common organic and inorganic compounds were of reagent grade and were used without further purification.

2.2 MAINTENANCE AND PREPARATION OF EHRLICH ASCITES CELLS

The Ehrlich ascites carcinoma was grown in male Swiss white mice, weighing 20 to 25 grams, and perpetuated by weekly intra-peritoneal injection of the ascitic suspension.

Mice carrying the tumor for 6 to 9 days were killed by cervical dislocation. An incision was then made in the abdominal wall and the cell suspension transferred to conical centrifuge tubes kept in an ice-water mixture. The cells were washed two or three times with 10 volumes of ice-cold isotonic saline to remove the ascitic fluid and any blood present. In washing, it was possible to sediment the tumor cells while leaving red blood cells in suspension by spinning the mixed suspension at 800 x g for only 15 to 20 seconds. If initially free of blood, the suspension was spun at 800 x g for 1 minute. After two or three washes, the cells were packed for 2 minutes at 800 x g. Only packed cells free of blood elements were subsequently used for experimentation.

2.3 GENERAL PREINCUBATION AND INCUBATION PROCEDURES

Cells were usually preincubated under various conditions to adjust the cellular levels of Na⁺, K⁺, ATP and amino acids.
They were subsequently incubated, again under various conditions, and cellular amino acid, Na\(^+\), K\(^+\) and ATP levels were estimated. Specific details of experimental design and the composition of preincubation and incubation media are documented in the relevant sections of the text. The general handling of the cells is described below.

Wet packed cells, prepared as in section 2.2 above, were resuspended in 10 volumes of ice-cold isotonic solution. 1 volume of this suspension was used per 2 volumes of medium. Preincubations were performed at 10\(^\circ\)C or 37\(^\circ\)C for 30 to 120 minutes. At the end of the preincubation period, the suspension was spun for 1 minute at 800 x g, the supernatants discarded, and the pellets packed for a further 2 minutes at 800 x g. The cells were resuspended and added to the incubation medium. The final dilution of cells was identical to that used in the preincubation: 1 volume of packed cells to 30 volumes of medium. Incubations were performed at 0\(^\circ\), 5\(^\circ\), 10\(^\circ\), 15\(^\circ\), 25\(^\circ\) and 37\(^\circ\)C for 1 to 120 minutes. Both preincubations and incubations were performed in Erlenmeyer flasks clamped in a shaking water bath. The preincubation or incubation medium consisted of a Ca\(^{++}\)-free Krebs-Ringer solution containing 145 mM NaCl, 5.8 mM KCl, 1.5 mM MgSO\(_4\), 1.5 mM K\(_2\)HPO\(_4\), 5 or 10 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonate (Hepes) pH 7.4, and various additions such as \(^{14}\)C-amino acids, DNP, glucose, etc. In numerous instances, the solution was modified: for example, the NaCl has often been replaced with isoosmotic amounts of KCl, choline chloride or mannitol.
Samples of the preincubation or incubation suspensions were removed for analysis at various times. They were placed in tared centrifuge tubes containing ice-cold isotonic saline and immediately spun at 800 x g for 1 minute. The supernatants were discarded, the pellets packed for an additional 2 minutes, at 800 x g, and the sides of the tubes dried with rolled tissue paper. The tubes were again weighed to obtain the weight of the packed cell sample which will henceforth be referred to as the sample "wet weight".

2.4 THE MEASUREMENT OF CELLULAR RADIOACTIVITY

Once the sample wet weight had been obtained, the cell pellets were resuspended in 2 ml of 85% ethanol or 5% trichloroacetic acid (TCA). The suspensions were allowed to stand for 20 minutes at room temperature, when ethanol was used, or on ice, when TCA was used. The ethanol and acid insoluble debris was pelleted for 2 minutes at 800 x g. The TCA was then removed from the TCA-extract supernatant by three extractions with 2.5 volumes of diethyl ether. Aliquots of the ethanol or acid soluble fractions were subsequently plated on aluminum planchette, evaporated to dryness under an infra-red lamp, and counted in a Nuclear Chicago thin end window gas-flow counter (Model No. 1042-8703) at 33% efficiency. As both extractions gave identical results, they were used interchangeably.

The incorporation of $^{14}$C-amino acid into cellular protein was estimated using a modification of the Schneider procedure (228).
The ethanol insoluble debris (from above) was twice washed with 95% ethanol and resuspended in 5% TCA. The suspension was heated for 30 minutes at 90°C, cooled, and the remaining acid insoluble debris pelleted at 800 x g for 2 minutes. The pellet was washed twice with 5% TCA, once with 95% ethanol and resuspended in ethanol-ether (3 to 1). The suspension was kept at 50°C for 15 minutes, cooled, and the remaining debris pelleted at 800 x g for 2 minutes. The pellet was washed once with ethanol-ether (3 to 1), and once with chloroform-ether (3 to 1). It was then resuspended in chloroform, plated on preweighed aluminum planchettes, allowed to dry at room temperature, weighed to obtain the dry protein weight and counted as above.

2.5 THE CALCULATION OF 14C-AMINO ACID UPTAKE

As estimated in section 2.4, the ethanol or acid soluble radioactivity represents that contained in the cells and in the extracellular fluid trapped between the cells of the sample pellet. The volume of that extracellular space was determined with (carboxyl-14C)inulin (M. Wt. 5000 to 5500) (55). The volume of the cell water compartment was calculated from the "inulin-space", and the wet and dry sample weights. Table 2-1 illustrates the distributions of extracellular and cellular water volumes in samples taken from a variety of incubation suspensions. It is apparent that while the cells swelled under some incubation conditions, the ratio of inulin-space to dry weight was fairly
TABLE 2-1

CELLULAR AND EXTRACELLULAR VOLUMES OF THE SAMPLE PELLET

Methods employed are described in sections 2.2 to 2.5 of this Chapter. The cells spent 20 to 60 minutes at 37°, 25°, or 10°C in a Ringer solution of specified composition containing (carboxyl-\(^{14}\)C) inulin (150 dpm per \(\mu\)l of medium). Estimation of the inulin space using 5% TCA, 85% ethanol or water extraction gave identical results. Values are given as means ± S.D. of at least 4 experimental observations.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Temp. (°C)</th>
<th>Inulin Space ((\mu)l/mg dry wt)</th>
<th>Cell Water ((\mu)l/mg dry wt)</th>
<th>Wet Weight (mg/mg dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krebs-Ringer</td>
<td>37</td>
<td>2.0 ± 0.1</td>
<td>3.7 ± 0.1</td>
<td>6.9 ± 0.5</td>
</tr>
<tr>
<td>Krebs-Ringer</td>
<td>25</td>
<td>1.9 ± 0.3</td>
<td>3.6 ± 0.4</td>
<td>6.8 ± 0.4</td>
</tr>
<tr>
<td>Krebs-Ringer + (10^{-4}) M DNP</td>
<td>25</td>
<td>1.9 ± 0.2</td>
<td>5.9 ± 0.4</td>
<td>8.8 ± 0.7</td>
</tr>
<tr>
<td>Krebs-Ringer</td>
<td>10</td>
<td>2.1 ± 0.2</td>
<td>6.7 ± 0.8</td>
<td>9.8 ± 0.8</td>
</tr>
<tr>
<td>Choline(^+)-Ringer</td>
<td>37</td>
<td>2.0 ± 0.1</td>
<td>3.7 ± 0.1</td>
<td>6.9 ± 0.5</td>
</tr>
<tr>
<td>Choline(^+)-Ringer</td>
<td>25</td>
<td>2.0 ± 0.2</td>
<td>3.7 ± 0.4</td>
<td>6.5 ± 0.6</td>
</tr>
<tr>
<td>Choline(^+)-Ringer + (10^{-4}) M DNP</td>
<td>25</td>
<td>2.2 ± 0.2</td>
<td>4.8 ± 0.6</td>
<td>7.9 ± 0.8</td>
</tr>
<tr>
<td>Choline(^+)-Ringer</td>
<td>10</td>
<td>1.9 ± 0.1</td>
<td>6.8 ± 0.7</td>
<td>9.7 ± 0.6</td>
</tr>
<tr>
<td>a) Choline(^+)-Ringer</td>
<td>37</td>
<td>2.0 ± 0.1</td>
<td>3.7 ± 0.1</td>
<td>6.9 ± 0.5</td>
</tr>
<tr>
<td>a) Choline(^+)-Ringer</td>
<td>25</td>
<td>2.0 ± 0.2</td>
<td>3.7 ± 0.4</td>
<td>6.5 ± 0.6</td>
</tr>
<tr>
<td>a) Choline(^+)-Ringer + (10^{-4}) M DNP</td>
<td>25</td>
<td>2.2 ± 0.2</td>
<td>4.8 ± 0.6</td>
<td>7.9 ± 0.8</td>
</tr>
<tr>
<td>a) Choline(^+)-Ringer</td>
<td>10</td>
<td>1.9 ± 0.1</td>
<td>6.8 ± 0.7</td>
<td>9.7 ± 0.6</td>
</tr>
<tr>
<td>b) K(^+)-Ringer + (10^{-4}) M DNP</td>
<td>25</td>
<td>2.2 ± 0.1</td>
<td>6.2 ± 0.5</td>
<td>8.9 ± 0.9</td>
</tr>
<tr>
<td>b) K(^+)-Ringer</td>
<td>10</td>
<td>1.9 ± 0.2</td>
<td>7.1 ± 0.3</td>
<td>9.8 ± 1.1</td>
</tr>
</tbody>
</table>

a) Modified Ringer solution wherein choline\(^+\) totally replaced Na\(^+\).

b) Modified Ringer solution wherein K\(^+\) totally replaced Na\(^+\).
constant. The correction for radioactivity trapped in the extracellular space could therefore be made knowing the sample wet and dry weights. The inulin-space reported here was found to be similar to that observed by Heinz and Mariani (55).

The cellular level of $^{14}$C-amino acid was calculated according to one of the following formulae.

$$\frac{\text{(total ethanol soluble dpm - trapped extracellular dpm)}}{\text{(specific activity of }^{14}\text{C-amino acid) } \times \text{(cell water volume)}} \quad \ldots \ A$$

$$\frac{\text{(total ethanol soluble dpm - trapped extracellular dpm)}}{\text{(specific activity of }^{14}\text{C-amino acid) } \times \text{(dry sample wt.)}}} \quad \ldots \ B$$

A has units of μmoles per ml cell water and B of nmoles per mg dry weight. The latter is used when comparing uptakes into swollen and unswollen cells.

2.6 THE MEASUREMENT OF EXCHANGE DIFFUSION

Cells were prepared (section 2.2) and divided into three portions for preincubation with L-(Me-$^{14}$C)methionine, L-methionine or without methionine. After a 40-45 minute preincubation period, the cells were transferred to the incubation medium wherein they would undergo exchange diffusion. Cells pretreated with L-(Me-$^{14}$C)methionine were used to estimate the exchangeable cellular methionine at time zero, thus serving as the "prepack controls". The remaining cells (those pretreated with and without L-methionine) were incubated with 2 mM L-(Me-
$^{14}$C)methionine (specific activity, 210 dpm/nmole). The uptake of radioactivity was estimated with time (section 2.4). The exchange process was presumed complete when the uptake of radioactivity -- corrected for the uptake not due to preloaded methionine -- attained at least 90% of the level of radioactivity observed in the "prepack controls". Uptake not due to preloaded methionine was determined by measuring the uptake of radioactivity into the cells pretreated in the absence of any methionine.

2.7 MEASUREMENT OF THE ENDOGENOUS FREE AMINO ACID POOL

Cells were prepared, preincubated, incubated and sampled as in sections 2.2 to 2.4. The sample pellets were extracted with 5% TCA and portions of the "deacidified" extracts were evaporated to dryness at 45°C under vacuum. Each residue was redissolved in 0.2 to 0.4 ml of water and applied to the top of a Technicon Chromobeads Type C-2 cation exchange resin column (0.6 by 67 cm). The amino acids in the sample were automatically chromatographed and their amounts estimated by the Technicon Amino Acid Autoanalyzer System.

2.8 THE MEASUREMENT OF $\text{Na}^+$ AND $\text{K}^+$

Cells were prepared, preincubated, incubated and sampled as in sections 2.2 to 2.4 except that the tubes into which the samples were placed did not contain saline. The cell samples were each digested with 0.4 ml of concentrated nitric acid at
40°C until the digest became clear (1 to 2 hours). The digest was diluted to 10.0 ml with water and filtered through Whatman No. 40 paper into plastic tubes. The Na⁺ in this solution was determined at 589 μm using a Zeiss PMQ II flame photometer. To determine K⁺, an aliquot of the filtered extract was removed and to it was added 0.25 volumes of M NaCl. K⁺ was determined in this solution at 770 μm. The values thus obtained represented the total of both cellular and extracellular cations. Correction for extracellular cations was made (section 2.5).

2.9 THE MEASUREMENT OF ATP

Cells were prepared, preincubated, incubated and sampled as in sections 2.2 to 2.4. They were extracted with 5% TCA and the "deacidified" extracts were adjusted to neutrality using small amounts of sodium bicarbonate. 1.5 ml aliquots of these solutions were used for the assay which was performed in incubation mixtures containing 150 μmoles of glycyl-glycine pH 7.4, 15 μmoles of MgCl₂, 30 μmoles of glucose, 3 μmoles of TPN⁺, 1.5 units of glucose-6-phosphate dehydrogenase and 0.14 units of hexokinase in a final volume of 3.0 ml. Two 1.5 ml aliquots from each cell sample were assayed; one in the absence and one in the presence of hexokinase. The increase in O.D. at 340 μm, due to the reduction of TPN⁺, in the presence of hexokinase over that in its absence is directly proportional to the amount of ATP present. A change in the optical density of 0.16 units was
equivalent to the reduction of 0.1 μmole of TPN⁺. This method is a modification of those used by Slein et al (229) and Nigam (230).
3.1 THEORETICAL

Johnstone and Scholefield (84, 92) have demonstrated that exchange diffusion of amino acids in Ehrlich ascites cells is insensitive to the removal of extracellular Na\(^+\). However, the effect of, (a), extracellular K\(^+\), (b), cellular Na\(^+\) and K\(^+\) and (c), transmembrane gradients of these cations on the process of amino acid exchange diffusion had not been investigated. The work reported herein was undertaken to examine the behavior of the exchange process under selected cellular energy states and ionic conditions. The \textit{a priori} hope was that the ensuing data would be of value in the elucidation of the role and degree of participation of exchange diffusion in the more general phenomenon of amino acid translocation across the plasma membrane of the Ehrlich ascites cell.

The basic procedure which was applied to estimate the exchange diffusion of methionine has been outlined in section 2.6 of Chapter 2. Any deviation from that procedure is fully documented in the relevant subsections of this chapter.

The rate constant, \(k\), for methionine homoexchange diffusion was calculated from the following equation

\[
\log_{10}(U_0-U_e) = \log_{10}U_0 - \frac{k}{2.303} t \quad \text{...........(3-1)}
\]
where $U_0$ is the exchangeable cellular methionine concentration at zero time and $U_e$ is the uptake of methionine due to exchange at any time, $t$. A plot of $\log_{10}(U_0-U_e)$ against $t$ will be linear, the slope being equal to $-k/2.303$ from which the numerical value of $k$ may be determined. $k$ will have units of $\text{MIN}^{-1}$.

The effect of temperature on the above rate constant may be described by the Arrhenius equation,

$$k = Ae^{-E/RT}$$

where $k$ is again the rate constant, $A$ is an integration constant, $e = 2.718$, $E$ is the "energy" of activation, $R$ is the gas constant ($1.987$ calories/degree/mole), and $T$ is the absolute temperature. The above equation can be rendered more useful by taking the $\log_{10}$ of both sides;

$$\log_{10}k = \log_{10}A - \frac{E}{2.303R} \cdot \frac{1}{T} \quad \ldots \ldots \ldots (3-2)$$

A plot of $\log_{10}k$ against $1/T$ will be linear and exhibit a slope equal to $-E/2.303R$ from which the value of $E$ may be determined. A more direct method of calculating the numerical value of $E$ requires the measurement of the rate constant, $k$, at two temperatures, $T_1$ and $T_2$. Then it can be shown that

$$E = 2.303R \frac{T_1T_2}{(T_2-T_1)} \log_{10} \frac{k_2}{k_1} \quad \ldots \ldots \ldots (3-3)$$

$E$ will have units of calories/mole.
A second method of describing the effect of temperature on the rate constant -- one which is more frequently employed in the biological sciences -- is the $Q_{10}$; the factor by which the reaction rate, $v$, increases due to a 10°C rise in temperature.

$$Q_{10} = \frac{v(T+10)}{v(T)} \quad \ldots \ldots \ldots (3-4)$$

### 3.2 THE EFFECT OF TEMPERATURE

Exchange diffusion of amino acids in Ehrlich ascites cells is known to occur rapidly even at room temperature (60). Furthermore, the establishment of normal cation distributions at 37°C also occurs with extreme rapidity unless metabolic poisons are added to the incubation medium (231,232). It was possible that the use of low temperatures would allow the measurement of exchange diffusion over a reasonable period of time (20 to 30 minutes) during which unusual cation distributions were maintained between cell and medium in the absence of metabolic inhibitors. The effect of temperature on the exchange process was therefore examined. Figure 3-1 shows the effect of temperature on the uptake of L-(Me-14C)methionine due to exchange. The data in Figure 3-1 were replotted according to equation No. 3-1 and appear in Figure 3-2. Comparison of the times necessary to achieve 90% completion of exchange at any temperature indicated that as far as the exchange diffusion of methionine was concerned, 10°C was the temperature best suited to the aforementioned
FIGURE 3-1. EFFECT OF TEMPERATURE ON METHIONINE EXCHANGE DIFFUSION. Methods employed are described in section 2.6 of Chapter 2. The incubation medium was a modified Ringer solution wherein isoosmotic amounts of mannitol replaced the NaCl and contained 2 mM L-(Me-14C)methionine, specific activity, 210 dpm/nmole. The open symbols represent the total uptake of L-(Me-14C)methionine into cells preloaded with methionine while the closed symbols correspond to the uptakes in cells pretreated in the absence of methionine.
FIGURE 3-2. EFFECT OF TEMPERATURE ON METHIONINE EXCHANGE DIFFUSION. Conditions as in Figure 3-1. All the lines in this Figure represent the total uptakes of L-(Me-14C)methionine into cells preloaded with methionine.
approach. Further support for the use of this temperature came from an examination of the cellular Na\(^+\) and K\(^+\) concentrations during the exchange process at 10\(^\circ\)C. Figure 3-3 shows the cellular Na\(^+\) and K\(^+\) concentrations during the exchange process which took place at 10\(^\circ\)C in a normal Krebs-Ringer medium. The plot demonstrated that even though extracellular Na\(^+\) and K\(^+\) concentrations were 145 mM and 8 mM respectively, the cellular levels of these ions were maintained at 40-60 mM and 80-60 mM respectively. All subsequent exchange experiments were therefore performed at 10\(^\circ\)C. Figure 3-4 is an Arrhenius plot which illustrates the effect of temperature on the rate constant for methionine homoechange diffusion according to equation No. 3-2. Using equation No. 3-3, the apparent activation energy for the exchange process was found to be 40,000 calories/mole. As defined by equation No. 3-4, the \(Q_{10}\) between 0 and 10\(^\circ\)C was 11.7 and that between 5 and 15\(^\circ\)C was 13.8.

3.3 THE EFFECT OF Na\(^+\) AND K\(^+\)

Examination of the effects of varying the ionic composition on both sides of the membrane began with a preincubation period during which the cells would simultaneously be preloaded with methionine and have their ionic contents suitably adjusted. This was accomplished by incubating the cells with various concentrations of L-methionine, Na\(^+\), K\(^+\), and choline\(^+\) at 10\(^\circ\)C and 37\(^\circ\)C for a 40-45 minute period. L-methionine was added to the
FIGURE 3-3. CELL Na\(^+\) AND K\(^+\) LEVELS DURING METHIONINE EXCHANGE DIFFUSION AT 10\(^\circ\)C. Methods employed are described in sections 2.6 and 2.8 of Chapter 2. The incubation medium was a normal Krebs-Ringer solution containing 2 mM L-methionine.
FIGURE 3-4. EFFECT OF TEMPERATURE ON THE RATE CONSTANT FOR
METHIONINE EXCHANGE DIFFUSION. Conditions as in Figure 3-2.
All the points represent the rate constants as determined from
the slopes of the lines in Figure 3-2.
preincubation media in concentrations sufficient to result in a cellular level of 12-15 mM of that amino acid. The variations in the extracellular Na\(^+\) and K\(^+\) concentrations and the resultant cellular Na\(^+\) and K\(^+\) concentrations, at both 10\(^\circ\)C and 37\(^\circ\)C, are outlined in Table 3-1. The table reveals that the preincubation was successful in generating methionine-loaded cells containing low (2-10 mM), normal (20-30 mM), and high (\(\geq 130\) mM) levels of Na\(^+\), as well as moderately low (approximately 20 mM), intermediate (80-120 mM) and high (\(\geq 180\) mM) cellular K\(^+\) concentrations. The preincubation conditions used did not yield cells containing less than 10 mM K\(^+\) or K\(^+\) levels in the range of 130-150 mM.

The cells of known Na\(^+\), K\(^+\) and methionine content were resuspended in fresh media containing 2 mM L-(Me-\(^{14}\)C)methionine and concentrations of mannitol, Na\(^+\) and K\(^+\) necessary to create a variety of transmembrane gradients in order to assess the effects of varying the ionic composition on both sides of the membrane on the exchange process. Table 3-2 shows values of the rate constant for homoexchange diffusion of methionine at 10\(^\circ\)C determined for cells of various intra- and extra-cellular Na\(^+\) and K\(^+\) concentrations. It is apparent that the rate constant fluctuated somewhat from day to day thus necessitating a comparison of the exchange process in all media listed in Table 3-2. Therefore, cells preincubated at 37\(^\circ\)C for 40-45 minutes in Krebs-Ringer containing 2 mM L-methionine were allowed to exchange in all media listed in Table 3-2. The rate constants
TABLE 3-1

VARIATION OF CELLULAR Na⁺ AND K⁺ CONCENTRATIONS DUE TO CHANGES IN THE COMPOSITION AND TEMPERATURE OF THE MEDIUM

Methods employed are described in section 2.8 of Chapter 2. The cells spent 40 to 45 minutes at 10°C or 37°C in solutions of specified composition. The values given are means ± S.D. of at least 3 experimental observations.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Na⁺ (μeq/ml)</th>
<th>Extracellular K⁺ (μeq/ml)</th>
<th>Choline⁺ (μmoles/ml)</th>
<th>Cellular Na⁺ (μeq/ml cell water)</th>
<th>Cellular K⁺ (μeq/ml cell water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>145</td>
<td>8</td>
<td>Nil</td>
<td>23 ± 6</td>
<td>185 ± 18</td>
</tr>
<tr>
<td></td>
<td>0-5</td>
<td>8</td>
<td>145</td>
<td>9 ± 1</td>
<td>119 ± 4</td>
</tr>
<tr>
<td></td>
<td>153</td>
<td>Nil</td>
<td>Nil</td>
<td>35*</td>
<td>80*</td>
</tr>
<tr>
<td>10</td>
<td>145</td>
<td>8</td>
<td>Nil</td>
<td>130 ± 16</td>
<td>80 ± 18</td>
</tr>
<tr>
<td></td>
<td>0-5</td>
<td>150</td>
<td>Nil</td>
<td>3 ± 1</td>
<td>200 ± 0</td>
</tr>
<tr>
<td></td>
<td>153</td>
<td>Nil</td>
<td>Nil</td>
<td>200*</td>
<td>20*</td>
</tr>
</tbody>
</table>

* One observation only.
**TABLE 3-2**

**Na⁺, K⁺ AND EXCHANGE DIFFUSION OF METHIONINE**

Methods employed are described in sections 2.6 and 2.8 of Chapter 2. The medium contained 2 mM L-(Me-14C) methionine-specific activity 210 dpm/nmole. Exchange was measured for 30 minutes at 10°C.

<table>
<thead>
<tr>
<th>Na⁺ (μeq/ml)</th>
<th>Extracellular Mannitol (μmoles/ml)</th>
<th>K⁺ (μeq/ml)</th>
<th>Cellular Na⁺ (μeq/ml cell water) t₀ t₃₀</th>
<th>Cellular K⁺ (μeq/ml cell water) t₀ t₃₀</th>
<th>k (minutes⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>145</td>
<td>Nil</td>
<td>8</td>
<td>41 61</td>
<td>86 66</td>
<td>0.067</td>
</tr>
<tr>
<td></td>
<td></td>
<td>121 107</td>
<td></td>
<td>30 27</td>
<td>0.068</td>
</tr>
<tr>
<td>153</td>
<td>Nil</td>
<td>Nil</td>
<td>41 -</td>
<td>90 -</td>
<td>0.043</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 -</td>
<td></td>
<td>160 -</td>
<td>0.041</td>
</tr>
<tr>
<td></td>
<td></td>
<td>160 -</td>
<td></td>
<td>10 -</td>
<td>0.045</td>
</tr>
<tr>
<td>5-10</td>
<td>290</td>
<td>8</td>
<td>26 19</td>
<td>129 100</td>
<td>0.077</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 8</td>
<td></td>
<td>104 85</td>
<td>0.073</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 0</td>
<td></td>
<td>117 80</td>
<td>0.081</td>
</tr>
<tr>
<td>5-10</td>
<td>290</td>
<td>8</td>
<td>26 -</td>
<td>130 -</td>
<td>0.060</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 -</td>
<td></td>
<td>202 -</td>
<td>0.061</td>
</tr>
<tr>
<td></td>
<td></td>
<td>190 -</td>
<td></td>
<td>42 -</td>
<td>0.063</td>
</tr>
<tr>
<td>5-10</td>
<td>Nil</td>
<td>153</td>
<td>0 0</td>
<td>248 248</td>
<td>0.055</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 0</td>
<td></td>
<td>235 235</td>
<td>0.055</td>
</tr>
<tr>
<td></td>
<td></td>
<td>69 47</td>
<td></td>
<td>100 100</td>
<td>0.054</td>
</tr>
</tbody>
</table>
for exchange averaged $0.067 \pm 0.003 \text{ MIN}^{-1}$ (S.D.) and therefore indicated that the differences in rate constant quoted in Table 3-2 were due to day to day variations in cellular activity. Further support of this conclusion came from Figure 3-4 which indicates that slight variations in temperature ($10^\circ \pm 1^\circ C$) can cause rather sizable variations in the rate constant for the exchange process ($0.073 \pm 0.015 \text{ MIN}^{-1}$). The results in Table 3-2 demonstrate that in any experiment, the rate constant was unaffected by the presence or absence of monovalent cations or their gradients.

### 3.4 THE EFFECT OF CELLULAR ATP

Heinz and Walsh (56) proposed that only a carrier preactivated by ATP could participate in the exchange diffusion of amino acids. This hypothesis was tested in the following manner. Cells were first preincubated in amino acid-free media, in the presence and absence of $10^{-4}$ M DNP for 45 minutes at $37^\circ C$. The DNP treated cells could no longer concentrate methionine -- even after prolonged incubation periods -- and contained not more than 0.1 mM ATP: the carrier, as visualized by Heinz and Walsh, was presumed to have been inactivated. However, those cells preincubated in the absence of the uncoupler could concentrate methionine well and contained roughly 2.5 mM ATP. Both lots of cells were then incubated for a further 45 minutes at $37^\circ C$ in fresh media, in the presence and absence of $10^{-4}$ M DNP as above, but
FIGURE 3-5. EFFECT OF ATP ON METHIONINE EXCHANGE DIFFUSION AT 10°C. Methods employed are described in section 2.6 of Chapter 2. The experimental design is described in section 3.4 of this Chapter. The incubation medium was a modified Ringer solution wherein isoosmotic amounts of mannitol replaced the NaCl and containing 2 mM L-(Me-14C)methionine, specific activity, 210 dpm/nmole. The solid symbols represent the uptake of L-(Me-14C)-methionine due to exchange in cells containing ATP while the hollow symbols represent the exchange in ATP-depleted cells.
containing quantities of methionine sufficient to generate 10-12 mM cellular methionine. The DNP-treated cells had thus acquired their amino acid through energy-independent means and should not have been capable of mediating methionine homoeexchange diffusion if that process required an "activated" carrier. Figure 3-5 shows that the uptake of L-(Me-14C) methionine due to exchange in cells with an inactive carrier and containing no more than 0.1 mM ATP was identical to that exhibited by the control cells. The same data plotted according to equation No. 3-1 appears in the insert. From the slope of that line, the rate constant for the exchange process was found to be 0.075 MIN⁻¹. It was therefore concluded that the carrier mediating methionine homoeexchange diffusion did not require preactivation with ATP.

3.5 SUMMARY

Homoexchange diffusion of methionine was found to be highly sensitive to temperature in the range between 0°C and 15°C. It was concluded that 10°C was the lowest possible temperature which allowed accurate estimation of the exchange process and the maintenance of unusual cation distributions between cell and medium over a 30 minute period. All subsequent exchange experiments were therefore performed at that temperature.

Variation of the extracellular Na⁺, K⁺, and choline⁺ concentrations during the 10°C or 37°C preincubation period yielded cells containing various known levels of Na⁺ and K⁺.
The rate constant for homoexchange diffusion of methionine was unaffected by the presence or absence of cellular ATP and monovalent cations or ionic gradients.

The carrier mediating the homoexchange diffusion of methionine does not require preactivation with cellular ATP.
CHAPTER 4
THE UPTAKE OF METHIONINE IN THE ABSENCE AND
PRESENCE OF EXTRACELLULAR Na+

4.1 THEORETICAL

Although the experiments in the previous Chapter demonstrated that exchange diffusion was active in cells preloaded with amino acid, they were not designed to reveal, (a), how much exchange occurs in cells not preloaded with amino acid and (b), whether the exchange carrier can mediate net uptake. Since the removal of extracellular Na+ is known to decrease amino acid transport (57,92) but is without effect on methionine exchange diffusion (92, Chapter 3), the examination of methionine uptake in the absence and presence of extracellular Na+ was undertaken to estimate the contribution of the exchange process to the total methionine uptake.

The procedures employed to estimate the uptake of L-(Me-14C)-methionine have been outlined in sections 2.1 to 2.5 of Chapter 2. The theoretical basis for the kinetic analysis used in this Chapter has been discussed in section 1.2.A. of Chapter 1.

4.2 THE ENDOGENOUS FREE AMINO ACID POOL

The contribution of the Na+-insensitive exchange mediation to the total methionine uptake would be expected to become a greater fraction of the total uptake as extracellular Na+ was
removed. Furthermore, as Oxender (159) has demonstrated that Ehrlich ascites cells contain an endogenous pool of free amino acids, a source of material capable of participating in exchange diffusion with extracellular methionine is available. The uptake of L-(Me-\(^{14}\))C)-methionine in Na\(^+\)-free medium can be compared to simultaneous changes in the composition of the endogenous pool of free amino acids brought about by the presence of methionine, thus estimating the uptake of methionine due to amino acid exchange diffusion. Knowing the total methionine uptake and the contribution of the exchange process, the relative contribution of exchange diffusion to the total uptake can be determined. The composition of the endogenous free amino acid pool was first examined after pretreating and incubating the cells under various conditions. The results are listed in Table 4-1 and indicate that the pool size decreases with the time of incubation. The presence of 10\(^{-4}\) M DNP in the medium resulted in almost complete depletion of the pool. To estimate the contribution of exchange diffusion to the total methionine uptake, cells were pretreated for 40 minutes in normal Krebs-Ringer at 37\(^\circ\)C, then transferred to a modified Ringer medium in which choline\(^+\) replaced the Na\(^+\), for one hour at 25\(^\circ\)C. Half the cells were incubated in an amino acid-free medium, the other half in a medium containing 2 mM L-(Me-\(^{14}\))C)methionine. Table 4-2 illustrates the changes in the cellular free amino acid pool that occurred due to the presence of 2 mM L-(Me-\(^{14}\))C)methionine extracellularly, and compares the changes in the endogenous pool to
TABLE 4-1

COMPOSITION OF THE ENDOGENOUS FREE AMINO ACID POOL

Methods employed are described in section 2.7 of Chapter 2. The temperature of incubation was 37°C in each case.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Krebs-Ringer (30 sec)</th>
<th>Krebs-Ringer (40 min)</th>
<th>Krebs-Ringer 40 min. then Choline⁺-Ringera (2 min)</th>
<th>Choline⁺-Ringera (60 min)</th>
<th>K⁺-Ringer+DNPb (60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>1.23</td>
<td>1.98</td>
<td>1.61</td>
<td>2.14</td>
<td>0.02</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.19</td>
<td>0.86</td>
<td>1.10</td>
<td>0.61</td>
<td>0.03</td>
</tr>
<tr>
<td>Serine</td>
<td>0.72</td>
<td>0.32</td>
<td>0.40</td>
<td>0.17</td>
<td>0.03</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>6.57</td>
<td>4.93</td>
<td>4.22</td>
<td>1.90</td>
<td>0.08</td>
</tr>
<tr>
<td>Proline</td>
<td>--</td>
<td>1.75</td>
<td>--</td>
<td>1.05</td>
<td>--</td>
</tr>
<tr>
<td>Glycine</td>
<td>6.34</td>
<td>3.54</td>
<td>3.57</td>
<td>3.02</td>
<td>0.39</td>
</tr>
<tr>
<td>Alanine</td>
<td>7.39</td>
<td>4.95</td>
<td>3.54</td>
<td>1.62</td>
<td>0.13</td>
</tr>
<tr>
<td>Valine</td>
<td>0.72</td>
<td>0.39</td>
<td>0.54</td>
<td>0.26</td>
<td>0.05</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.11</td>
<td>0.30</td>
<td>0.15</td>
<td>0.09</td>
<td>--</td>
</tr>
<tr>
<td>Iso-leucine</td>
<td>0.28</td>
<td>0.18</td>
<td>0.23</td>
<td>0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.24</td>
<td>0.25</td>
<td>0.47</td>
<td>0.32</td>
<td>0.02</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.29</td>
<td>0.29</td>
<td>0.34</td>
<td>0.20</td>
<td>0.02</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.31</td>
<td>0.21</td>
<td>0.34</td>
<td>0.18</td>
<td>0.02</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.06</td>
<td>0.34</td>
<td>--</td>
<td>--</td>
<td>0.04</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.51</td>
<td>0.20</td>
<td>--</td>
<td>--</td>
<td>0.02</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.18</td>
<td>0.14</td>
<td>--</td>
<td>--</td>
<td>0.01</td>
</tr>
</tbody>
</table>

a A modified Ringer solution wherein choline⁺ replaced the Na⁺.

b A modified Ringer solution wherein K⁺ replaced the Na⁺ and containing 10⁻⁴ M DNP.
TABLE 4-2

THE ENDOGENOUS POOL OF "EXCHANGEABLE" AND "NON-EXCHANGEABLE" AMINO ACIDS
IN THE PRESENCE AND ABSENCE OF ADDED METHIONINE

Methods employed are described in sections 2.2 to 2.5 and 2.7 of Chapter 2. The cells were pretreated at 37°C for 40 minutes in normal Krebs-Ringer medium. They were subsequently transferred to two modified Ringer solutions wherein choline+ replaced the Na+ and one of which contained 2 mM L-(Me-14C)-methionine -- specific activity 450 dpm/nmole.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Cellular amino acid pool levels (μmoles/ml cell water)</th>
<th>L-(Me-14C) methionine uptake (μmoles/ml cell water)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+methionine</td>
</tr>
<tr>
<td>Exchangeable Pool (a)</td>
<td>2</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.20</td>
</tr>
<tr>
<td>Non-Exchangeable Pool (b)</td>
<td>2</td>
<td>14.40</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>8.90</td>
</tr>
</tbody>
</table>

(a) The group of amino acids consisting of isoleucine, leucine, phenylalanine, tyrosine and valine.
(b) The group of amino acids consisting of alanine, aspartic and glutamic acids, glycine, serine and threonine.
the \( \text{L-(Me}_{\text{-14}} \text{C)} \) methionine uptake. The amino acids grouped in the category of the "exchangeable pool" are all known to participate in exchange diffusion with methionine while those in the class of the "non-exchangeable pool" do not significantly exchange with methionine (48,161). It is apparent from the Table that 90% of the \( \text{L-(Me}_{\text{-14}} \text{C)} \) methionine uptake after two minutes of incubation in \( \text{Na}^{+} \)-free medium could be balanced by a simultaneous loss of amino acids from the endogenous "exchangeable pool". However, the additional uptake between 2 and 60 minutes was not balanced by the loss of any amino acids from either pool. Furthermore, the total uptake of \( \text{L-(Me}_{\text{-14}} \text{C)} \) methionine resulted in an apparent cellular methionine concentration greater than that of the extracellular fluid. It was therefore concluded that the apparent initial rapid net uptake of \( \text{L-(Me}_{\text{-14}} \text{C)} \) methionine from \( \text{Na}^{+} \)-free medium was due largely to an exchange process between extracellular methionine and the amino acids comprising the endogenous "exchangeable pool" while the latter phase of uptake was primarily due to net transport activity. Furthermore, since the rate of methionine exchange diffusion was shown to be unaffected by \( \text{Na}^{+} \) (Table 3-2), it seemed reasonable to assume that methionine uptake due to exchange would be identical in the presence and absence of extracellular \( \text{Na}^{+} \). Then under the present experimental conditions, the contribution of exchange diffusion to the total initial uptake of \( \text{L-(Me}_{\text{-14}} \text{C)} \) methionine in the absence or presence of extracellular \( \text{Na}^{+} \) at \( 25^\circ \text{C} \) is 1.35 umoles/ml cell water.
This evidence does not, however, indicate whether the residual net methionine uptake in the absence of Na\(^+\) (that not accounted for by exchange diffusion with endogenous amino acids) is catalyzed by the carrier mediating the exchange diffusion of neutral amino acids. If the initial uptake of L-(Me-\(^{14}\)C)methionine in the absence of Na\(^+\) is due largely to an exchange process while the subsequent uptake is due to net transport catalyzed by a system other than the exchange carrier, then in the absence of Na\(^+\), (a), amino acids transported into these cells but having little or no affinity for the exchange carrier should not initially affect the uptake but should inhibit it at later times, (b), amino acids having affinity for the exchange carrier should inhibit the initial uptake and (c), kinetic analysis should indicate two components in the initial uptake. However, if the exchange carrier can catalyze net methionine uptake, then in a Na\(^+\)-free medium, cells depleted of ATP and their endogenous amino acid pools should not exhibit the rapid initial uptake attributable to exchange diffusion with amino acids of the endogenous pool, but should take up methionine from the medium until the cellular concentration of that amino acid equals that in the medium. The remaining subsections of this Chapter describe the results of experiments designed to test the predictions stated above.
4.3 THE EFFECT OF OTHER NEUTRAL AMINO ACIDS

The design of these experiments was based on the observed behavior of both the exchange diffusion and active transport systems in Ehrlich cells. It has been demonstrated that the uptake of methionine is mediated by more than one carrier system (48, 60, 75, 161) and is inhibitable by other neutral amino acids (84, 161). If, as suggested by Inui and Christensen (161), the methionine uptake in the absence of extracellular Na\(^+\) was mediated by the carrier catalyzing exchange diffusion (L system), then only amino acids with affinities for that carrier should inhibit the methionine uptake in the absence of Na\(^+\). Neutral amino acids were therefore chosen for this study according to their reputed affinities for either the A or L mediation which primarily catalyze the active transport or exchange diffusion of neutral amino acids respectively (48). Thus α-aminoisobutyric acid (α-AIB) and L-alanine were employed as representative of those neutral amino acids with strong affinities for the A mediation while L-leucine and L-valine served for those with high affinities for the L mediation. Furthermore, it has been shown that both α-AIB and L-alanine do not participate in the process of exchange diffusion with methionine to any significant degree nor do they inhibit exchange diffusion between methionine and various other amino acids (48). The effects of 10 mM concentrations of α-AIB and L-alanine on the uptake of methionine from normal Krebs-Ringer medium containing 2 mM L-(Me\(^-14\))C-
FIGURES 4-1A AND 4-1B. EFFECT OF OTHER NEUTRAL AMINO ACIDS ON METHIONINE UPTAKE AT 25°C. Methods employed are described in sections 2.2 to 2.5 of Chapter 2. Cells were preincubated for 40 minutes at 37°C in normal Krebs-Ringer medium. They were subsequently transferred to fresh Krebs-Ringer media containing 2 mM L-({\textsuperscript{14}C})methionine (specific activity, 450 dpm/nmole) and 10 mM concentrations of α-AIB, L-alanine, L-leucine or L-valine as indicated above. Figure 4-1A is on the left while Figure 4-1B is on the right.
FIGURES 4-2A AND 4-2B. EFFECT OF OTHER NEUTRAL AMINO ACIDS ON METHIONINE UPTAKE IN THE ABSENCE OF Na⁺ AT 25°C. Conditions were identical to those in Figures 4-1A and 4-2A except the incubation medium was a modified Ringer solution wherein choline⁺ replaced the Na⁺. Figure 4-2A is on the left while Figure 4-2B is on the right.
methionine over a 1 hour period at 25°C is shown in Figure 4-1A. The effects of 10 mM concentrations of L-leucine and L-valine are illustrated in Figure 4-1B. The results of parallel experiments performed in the absence of extracellular Na\(^+\) appear in Figures 4-2A and 4-2B. It is apparent that all four amino acids inhibited the methionine uptake in both the absence and presence of extracellular Na\(^+\). However, in the absence of Na\(^+\), the inhibition caused by leucine or valine was already apparent at 2 minutes of incubation while that caused by \(\alpha\)-AIB and alanine was not, only becoming apparent after 10 to 15 minutes of incubation. Furthermore, comparison of the initial rates of uptake in Figures 4-1A and 4-2A indicates that the residual component of the total initial uptake in the presence of Na\(^+\) and 10 mM \(\alpha\)-AIB or L-alanine (Figure 4-1A) was approximately equal in magnitude to the uptake in the absence of Na\(^+\) at the same time period (Figure 4-2A). These observations suggest that the initial uptake was due mostly to an exchange process which contributed as much methionine to the total uptake in the absence as in the presence of Na\(^+\). The exchange contribution estimated from the data in Figures 4-1A and 4-2A is 1.4 \(\mu\)moles/ml cell water and is in close agreement with that found in Table 4-2. It has been previously stated that Inui and Christensen (161) implied that the methionine uptake in the absence of Na\(^+\) was mediated by the carrier catalyzing exchange diffusion. This implication is entirely consistent with the results in Table 4-2,
Figure 4-1A and Figure 4-2A, but only so far as the initial rate of methionine uptake is concerned. Comparison of the uptakes in Figures 4-1A and 4-2A between 2 and 60 minutes of incubation revealed that not only did α-AIB and alanine inhibit methionine uptake in the absence of Na\(^+\), but that the α-AIB insensitive component in the presence of Na\(^+\) was no longer equal to the uptake in the absence of Na\(^+\). Furthermore, when the contribution of exchange diffusion from Table 4-2 was subtracted from each curve in Figures 4-1A and 4-2A, it was apparent that the residual uptakes in the absence and presence of Na\(^+\) between 2 and 60 minutes were both inhibited by 50% in the presence of 10 mM α-AIB. It therefore seemed likely that the uptake in the absence of Na\(^+\) which was not accounted for by exchange diffusion was not mediated by the exchange carrier (L system) but was due to the residual activity of the A transport system.

In conclusion, it can be stated that, (a), the initial methionine uptake in the absence of Na\(^+\) is due largely to exchange while the latter phase is not, (b), the contribution of the exchanging system to the total uptake in the presence or absence of Na\(^+\) was found to be 1.4 μmoles/ml cell water and (c), the component of methionine uptake in the absence of Na\(^+\) not attributable to exchange is probably due to the residual activity of the A system.
4.4 THE EFFECT OF EXTRACELLULAR METHIONINE CONCENTRATION

The results presented above indicated that in the absence of extracellular Na\(^+\), the total methionine uptake contained an \(\alpha\)-AIB sensitive as well as an \(\alpha\)-AIB insensitive component. It seemed likely from the \(\alpha\)-AIB inhibition studies that the \(\alpha\)-AIB sensitive, nonexchange component represents the residual activity of the A system. Furthermore, the data in Table 4-2 indicated that a small fraction of the initial methionine uptake in the absence of Na\(^+\) could not be accounted for by exchange processes. If, (a), that small fraction of uptake was the contribution of the A system and (b), the A system and the exchange carrier respond to changes in the extracellular methionine concentration to a sufficiently different degree, then the kinetics of methionine uptake with respect to extracellular methionine concentration should indicate the contributions of two processes in the absence of Na\(^+\). The response of methionine uptake to extracellular L-(Me-\(^{14}\)C)methionine concentration in the absence of extracellular Na\(^+\) is illustrated in Figure 4-3 according to the method of Lineweaver and Burk (117). It was evident from the plot that the uptake in the absence of Na\(^+\) appeared to be the result of two processes. The component represented by the line with the lesser slope was arbitrarily designated the X process while that with the greater slope was termed the Y process. The apparent Michaelis constants and maximum uptakes for these two processes are listed in Table 4-3. Because of the similarity
FIGURE 4-3. METHIONINE UPTAKE IN THE ABSENCE OF EXTRACELLULAR Na+. Methods employed are described in sections 2.2 to 2.5 of Chapter 2. The incubation medium was a modified Ringer solution wherein choline+ replaced the Na+. L-(Me-14C)-methionine was used at a specific activity of 390 dpm/nmole. The incubation was performed at 37°C. The uptakes after one minute of incubation are shown.
TABLE 4-3

KINETIC PARAMETERS FOR METHIONINE UPTAKE IN THE ABSENCE OF EXTRACELLULAR Na⁺

Methods and conditions are described in the legend of Figure 4-3.

<table>
<thead>
<tr>
<th>Component</th>
<th>Apparent $K_m$ (μmoles/ml)</th>
<th>Apparent $V_{max}$ (μmoles/ml cell water/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>0.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Y</td>
<td>0.5</td>
<td>2.2</td>
</tr>
</tbody>
</table>
between the magnitudes of the X component and the estimates of uptake attributed to exchange diffusion (Table 4-2), the X component was viewed as the contribution of exchange diffusion while the Y component was probably indicative of net transport.

4.5 THE EFFECT OF ATP DEPLETION

When the uptake of methionine is measured under conditions which would inhibit the activity of the net transport system, the contribution of the exchange carrier to the methionine uptake should be discernable. Net accumulation of amino acids requires ATP (45,75,84) and is stimulated by extracellular Na⁺ (57,79,92), while the carrier catalyzing amino acid exchange diffusion is insensitive to Na⁺, K⁺ and ATP (Chapter 3). Furthermore, it has been stated that cells pretreated in 10⁻⁴ M DNP contained no more than 0.1 mM ATP (section 3.4 of Chapter 3) and only a very small endogenous exchangeable amino acid pool (Table 4-1). Then if the exchange carrier can catalyze net methionine uptake, ATP- and amino acid- depleted cells should take up methionine from a Na⁺-free medium at least until the cellular and extracellular levels of that amino acid have equilibrated. Cells were therefore preincubated in a modified Ringer solution wherein K⁺ replaced the Na⁺ and containing 10⁻⁴ M DNP, for 60 minutes at 37°C. The cells were subsequently transferred to modified Ringer solutions wherein choline⁺ replaced the Na⁺ and containing L-(Me⁻¹⁴ C) methionine and 10⁻⁴ M DNP. In one case, 10 mM
METHODS employed are described in sections 2.2 to 2.5 and 2.9 of Chapter 2. The cells were pretreated at 37°C for 60 minutes in a modified Ringer solution wherein K+ replaced the Na+ and containing $10^{-4}$ M DNP. The cells were subsequently incubated at 25°C in a modified Ringer solution wherein choline replaced the Na+. The incubation medium contained L-(Me-14C)methionine (specific activity 360 dpm/nmole) and $10^{-4}$ M DNP except in one case where 10 mM glucose was added instead of the uncoupler.

<table>
<thead>
<tr>
<th>Extracellular L-(Me-14C)methionine (μmoles/ml)</th>
<th>Cellular ATP (μmoles/ml cell water)</th>
<th>Uptake of L-(Me-14C)methionine (μmoles/ml cell water/2 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>$&lt; 0.1$ a</td>
<td>0.4</td>
</tr>
<tr>
<td>2.0+20 mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-ethionine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.0</td>
<td>$&lt; 0.1$ a</td>
<td>0.2</td>
</tr>
<tr>
<td>2.0</td>
<td>$&gt; 0.1$ a</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>$&gt; 1.6$ b</td>
<td>2.1</td>
</tr>
</tbody>
</table>

a Incubation medium contained $10^{-4}$ M DNP.

b Incubation medium contained 10 mM glucose and no DNP (see Figure 5-1).
glucose was added to the medium instead of the uncoupler. The uptake of L-(Me-\textsuperscript{14}C)methionine was estimated over a 2 hour period. The results in Table 4-4 show that extracellular methionine does not equilibrate with the cellular fluid within a 2 hour period unless the cellular ATP concentration is elevated. Furthermore, half the uptake in the absence of ATP can be suppressed by L-ethionine suggesting that at least half this uptake is still carrier mediated. These results indicate that the carrier mediating exchange diffusion (L system) does not catalyze the net uptake of methionine.

4.6 SUMMARY

The uptake of methionine into ATP-containing cells appears to be the result of the operation of two processes: an initial, rapid, Na\textsuperscript{+}-insensitive exchange process between extracellular methionine and the endogenous exchangeable free amino acid pool, and a net transport process which was markedly inhibited by the removal of extracellular Na\textsuperscript{+}. The exchange process contributes approximately 1.4 \textmu moles/ml cell water of methionine to the total uptake. The net transporting system was operative in essentially Na\textsuperscript{+}-free medium but only at a fraction of the rate at which it functioned in normal Krebs-Ringer medium. Furthermore, the carrier mediating exchange diffusion (L system) does not appear to catalyze the net uptake of methionine.
CHAPTER 5

NEUTRAL AMINO ACID UPTAKE, ATP AND MONOVALENT CATION GRADIENTS

5.1 THEORETICAL

While examining the process of exchange diffusion at 10°C (Chapter 3), the uptake of methionine under various ionic conditions was also estimated. The results of preliminary experiments indicated that the presence of an inward Na⁺ gradient was not necessary for the transport of methionine. This was contrary to the so-called Na⁺ gradient hypothesis (99,103) which maintains that the asymmetric transmembrane distribution of Na⁺ and K⁺, rather than ATP, provides the required energy for the uphill carrier mediated transport of amino acids and sugars. The rationale of this hypothesis can be summarized as follows. Na⁺, which is a co-substrate in the uphill transport of amino acids and sugars, primarily increases the affinity of the amino acid or sugar carrier systems for their respective substrates. K⁺, however, decreases this affinity. Thus the inward, uphill transport of amino acids and sugars is observed in cells with an inward Na⁺ (and outward K⁺) gradient. Furthermore, most animal cells maintain a relatively low Na⁺ and high K⁺ concentration via the ATP-dependent Na⁺-pump mechanism. ATP is not directly required for the operation of the uphill amino acid or
sugar transport systems. If this model is correct, then experimenta-
tion should show that the net uphill flux of amino acids or sugars occurs in the same direction as that of the transmem-
brane Na\(^+\) gradient and depends on its magnitude rather than the level of cellular ATP. For instance, net uphill transport into cells with an inward Na\(^+\) gradient of normal magnitude should be unaffected by the level of cellular ATP. Furthermore, no net uphill transport should be observed when the Na\(^+\) gradient is abolished.

The experiments reported herein were undertaken to test these predictions and thus the applicability of the Na\(^+\) gradient hypothesis to the uphill transport of neutral amino acids by Ehrlich ascites cells. The experimental procedures have been described in sections 2.2 to 2.5 of Chapter 2. Any deviations from those procedures are fully documented in the relevant sub-sections of this Chapter.

5.2 ON THE NECESSITY OF THE Na\(^+\) GRADIENT

Although methionine uptake at 10\(^\circ\)C is small, it is measurable and consistent. Since it was possible to maintain abnormal ionic distributions at this temperature without resorting to the use of extraneous inhibitors (Table 3-2), it was also possible to estimate the dependence of methionine uptake on the Na\(^+\) gradient. If the Na\(^+\) gradient determines the direction of net amino acid movement, then the uptake of methionine should
be largest in cells with a sizable (normal) inward Na\(^+\) gradient and smallest in cells with a large (reversed) outward Na\(^+\) gradient. Thus, cells were first preincubated as described in section 3.3 of Chapter 3, to obtain appropriate cellular ion concentrations, and then transferred to fresh Krebs-Ringer medium or to modified Ringer solutions in which the sodium chloride was replaced with isoosmotic amounts of mannitol or choline chloride. The uptake of 2 mM L-(Me-\(^{14}\)C)methionine at 10\(^{0}\)C under the influence of the Na\(^+\) gradients thus created is illustrated in Table 5-1. The results of experiment 1 show that the uptake of methionine was not decreased when the inward Na\(^+\) gradient was essentially abolished provided that a normal level of extracellular Na\(^+\) was present. In the absence of extracellular Na\(^+\), the uptake was not decreased when the Na\(^+\) gradient was reversed. This evidence suggests that the uptake of methionine does not depend on the Na\(^+\) gradient. It should also be stated that the uptake of methionine at 10\(^{0}\)C is a carrier mediated process, the activity of which was reduced in the absence of extracellular Na\(^+\) (experiment 1) and in the presence of other amino acids (experiment 2). However, because the magnitude of the uptakes is small and the relative error inherent in their determination at this temperature is large, the investigation was continued at higher temperatures to increase the uptake and minimize the relative error.

The objectives of the following experiments were not only to corroborate the conclusions formulated on the basis of the
**TABLE 5-1**

THE $\text{Na}^+$ GRADIENT AND METHIONINE UPTAKE AT 10°C

Methods employed are described in sections 2.2 to 2.5 and 2.8 of Chapter 2. Cells were pretreated to obtain the appropriate cellular ion concentrations (see section 3.3 of Chapter 3). They were subsequently transferred to fresh Krebs-Ringer medium or to modified Ringer solutions in which the NaCl was replaced with mannitol (experiment 1) or choline chloride (experiment 2) and containing 2 mM $\text{L-(Me}^{14}\text{C)}$-methionine, specific activity 210 dpm/nmole (experiment 1), or 840 dpm/nmole (experiment 2). The data presented are averages obtained from at least two or three separate experiments. The magnitude of variations from one experiment to the next can be seen by comparing the uptakes in experiments 1 and 2 for 0-2 μeq/ml of extracellular $\text{Na}^+$.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Additions</th>
<th>Extracellular $\text{Na}^+$ (μeq/ml)</th>
<th>Cellular $\text{Na}^+$ $t_0$ (μeq/ml cell water)</th>
<th>Cellular $\text{Na}^+$ $t_{30}$ (μeq/ml cell water)</th>
<th>L-$\text{Me}^{14}\text{C)}$-methionine uptake (μmoles/ml cell water/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 mM L-methionine</td>
<td>145</td>
<td>41</td>
<td>61</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>2 mM L-methionine</td>
<td>145</td>
<td>121</td>
<td>107</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>2 mM L-methionine</td>
<td>0-2</td>
<td>0-5</td>
<td>0-5</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>2 mM L-methionine</td>
<td>0-2</td>
<td>26</td>
<td>19</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>2 mM L-methionine</td>
<td>0-2</td>
<td>135</td>
<td>80-100</td>
<td>1.4</td>
</tr>
<tr>
<td>2</td>
<td>2 mM L-methionine</td>
<td>0-2</td>
<td>25-30</td>
<td>0-5</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>+5 mM L-ethionine</td>
<td>0-2</td>
<td>25-30</td>
<td>0-5</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>+10 mM L-ethionine</td>
<td>0-2</td>
<td>25-30</td>
<td>0-5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>+10 mM L-valine</td>
<td>0-2</td>
<td>25-30</td>
<td>0-5</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>+10 mM L-alanine</td>
<td>0-2</td>
<td>25-30</td>
<td>0-5</td>
<td>0.9</td>
</tr>
</tbody>
</table>
observations at 10°C, but to extend the evidence and determine whether the uptake of amino acids was more sensitive to the cellular ATP level or to the Na⁺ gradient. Since methionine uptake was shown to be sensitive to the concentration of extracellular Na⁺ (92,161), the extracellular Na⁺ concentration was maintained at 145 mM while the transmembrane Na⁺ gradient was reduced or abolished by elevating the cellular Na⁺ concentration. This rise can be produced by preincubating the cells in normal Krebs-Ringer medium, (a), at low temperatures (Table 3-1) or (b), in the presence of metabolic inhibitors such as 2,4-DNP. Both methods result in cell swelling (Table 2-1).

The former alters the cellular ATP concentration but not the total amount of cellular ATP, while the latter alters both. When cells pretreated in DNP-containing media are transferred to fresh, inhibitor-free medium containing glucose, the cellular ATP level quickly approaches that of cells not pretreated in DNP. Figure 5-1 illustrates the effect of reincubation in the presence and absence of 10⁻⁴ M DNP and 10 mM glucose on the cellular ATP level of cells pretreated in the presence and absence of 10⁻⁴ M DNP. Methods are thus available for the independent variation of cellular Na⁺ and ATP concentrations. It was therefore possible to determine whether the uptake of amino acids was better correlated to the magnitude of the Na⁺ gradient or the cellular ATP level.

The results in Table 5-2 demonstrate that at 25°C, the uptake
FIGURE 5-1. CELL ATP LEVELS IN THE PRESENCE AND ABSENCE OF DNP AND GLUCOSE.
Methods employed are described in sections 2.2, 2.3 and 2.9 of Chapter 2. Cells were preincubated for 60 minutes at 37°C in normal Krebs-Ringer medium in the presence or absence of 10\(^{-4}\) M DNP. They were then transferred to fresh normal Krebs-Ringer media containing 2 mM L-methionine as well as the additions indicated above and kept at 25°C for 60 minutes. The hatched bars represent cells pretreated in DNP-containing medium while the open bars represent cells pretreated in the absence of DNP. At the end of the preincubation period in DNP, the cells contained 0.1 μmoles/ml cell water of ATP.
of L-(Me-\textsuperscript{14}C)methionine from normal Krebs-Ringer medium was much more sensitive to changes in the cellular ATP level than to changes in the magnitude of the inward Na\textsuperscript{+} gradient. Comparable accumulation of methionine was observed in cells containing ATP and either low or high levels of Na\textsuperscript{+} (see first 4 lines of Table 5-2). However, the uptake was reduced when the cells were depleted of ATP (see last 3 lines of Table 5-2). Similar results were observed with glycine (Table 5-3). The results in the first 2 lines of Table 5-4 show that cells containing ATP and low concentrations of Na\textsuperscript{+} will take up methionine to a greater degree when incubated in a Na\textsuperscript{+}-containing rather than a Na\textsuperscript{+}-free medium. The last 2 lines of the Table show that the same effect is observed with cells initially containing high concentrations of Na\textsuperscript{+}. Comparison of lines 1 and 3 or lines 2 and 4 of Table 5-4 reveals that the methionine uptake is relatively insensitive to the magnitude and direction of the transmembrane Na\textsuperscript{+} gradient. It is also apparent from Tables 5-2, 5-3, and 5-4 that in the presence of extracellular Na\textsuperscript{+}, comparable uptakes were observed in cells pretreated in the presence or absence of 10^{-4} M DNP provided that the cellular ATP was restored to levels near 2 mM. Extensive pretreatment in media containing 10^{-4} M DNP did not, therefore, result in irreversible damage to the systems transporting amino acids. Furthermore, the results in Table 5-5 demonstrate that protein synthesis was not required for the restoration of glycine transport activity.
**TABLE 5-2**

THE Na\(^+\) GRADIENT, ATP AND THE UPTAKE OF METHIONINE FROM NORMAL KREBS-RINGER MEDIUM

Methods employed are described in sections 2.2 to 2.5, 2.8 and 2.9 of Chapter 2. The cells were pre-incubated as indicated below for 60 minutes and subsequently transferred to normal Krebs-Ringer medium containing 2 mM L-(Me-\(^{14}\)C)methionine. The uptake of radioactivity, cellular Na\(^+\) and ATP were estimated for 30 minutes at 25°C.

<table>
<thead>
<tr>
<th>Preincubation Conditions</th>
<th>Medium Na(^+) (t_0) (μeq/ml)</th>
<th>Cellular Na(^+) (t_0) (μeq/ml cell water)</th>
<th>Cellular Na(^+) (t_0) (μmoles/ml cell water)</th>
<th>Cellular ATP (t_0) (μmoles/ml cell water)</th>
<th>Cellular ATP (t_0) (μmoles/ml cell water)</th>
<th>Cellular ATP (t_0) (μmoles/ml cell water)</th>
<th>Cellular ATP (t_0) (μmoles/ml cell water)</th>
<th>Cellular ATP (t_0) (μmoles/ml cell water)</th>
<th>Cellular ATP (t_0) (μmoles/ml cell water)</th>
<th>L-(Me-(^{14})C)methionine Uptake (t_0) (nmoles/mg dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C Krebs-Ringer</td>
<td>145</td>
<td>20-30</td>
<td>35-50</td>
<td>2.1</td>
<td>2.8</td>
<td>24.0</td>
<td>26.0</td>
<td>25.0</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td>10°C K(^+)-Ringer</td>
<td>145</td>
<td>2-4</td>
<td>35-50</td>
<td>2.1</td>
<td>2.8</td>
<td>24.0</td>
<td>26.0</td>
<td>25.0</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td>10°C Krebs-Ringer</td>
<td>145</td>
<td>170-180</td>
<td>140-160</td>
<td>2.3</td>
<td>3.1</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td>37°C Krebs-Ringer+DNP</td>
<td>145</td>
<td>155</td>
<td>110</td>
<td>1.6 b</td>
<td>1.9 b</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td>37°C Krebs-Ringer+DNP</td>
<td>145</td>
<td>155</td>
<td>145</td>
<td>0.3</td>
<td>0.4</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>37°C Krebs-Ringer+DNP</td>
<td>145</td>
<td>155</td>
<td>150</td>
<td>≤0.1 c</td>
<td>≤0.1 c</td>
<td>8.2</td>
<td>8.2</td>
<td>8.2</td>
<td>8.2</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) A cellular concentration of 2 μmoles/ml cell water of methionine is equivalent to 7.4 nmoles/mg dry wt. in the unswollen cell.

\(^b\) The incubation medium contained 10 mM glucose.

\(^c\) The incubation medium contained 10^{-4} M DNP.

The values above are means from 2 or 3 experiments which differed by no more than 10%.
Methods employed are described in sections 2.2 to 2.5, 2.8 and 2.9 of Chapter 2. The cells were pre-incubated as indicated below for 60 minutes, then transferred to normal Krebs-Ringer medium containing 2 mM (1-14C)glycine, specific activity 360 dpm/nmole. The uptake of glycine, cellular Na\(^+\) and ATP were estimated for 60 minutes at 37°C.

<table>
<thead>
<tr>
<th>Preincubation Conditions</th>
<th>Medium Na(^+) (t_0) (µeq/ml)</th>
<th>Cellular Na(^+) (t_0) (µeq/ml cell water)</th>
<th>Cellular Na(^+) (t_{60}) (µeq/ml cell water)</th>
<th>Cellular ATP (t_0) (µmoles/ml cell water)</th>
<th>Cellular ATP (t_{60}) (µmoles/ml cell water)</th>
<th>(1-14C)glycine Uptake (t_{60}) (nmoles/mg dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C Krebs-Ringer</td>
<td>145</td>
<td>20-30</td>
<td>---</td>
<td>3.5 (^b)</td>
<td>3.1 (^b)</td>
<td>82.1</td>
</tr>
<tr>
<td>37°C Krebs-Ringer+DNP</td>
<td>145</td>
<td>150-160</td>
<td>---</td>
<td>2.5 (^b)</td>
<td>2.7 (^b)</td>
<td>74.7</td>
</tr>
<tr>
<td>37°C Krebs-Ringer+DNP</td>
<td>145</td>
<td>150-160</td>
<td>---</td>
<td>0.2</td>
<td>0.4</td>
<td>23.0</td>
</tr>
<tr>
<td>37°C Krebs-Ringer+DNP</td>
<td>145</td>
<td>150-160</td>
<td>150-160</td>
<td>(\leq 0.1 (^c))</td>
<td>(\leq 0.1 (^c))</td>
<td>16.4</td>
</tr>
</tbody>
</table>

\(^a\) A cellular concentration of 2 µmoles/ml cell water of glycine is equivalent to 7.4 nmoles/mg dry wt. in the unswollen cell.

\(^b\) The incubation medium contains 10 mM glucose.

\(^c\) The incubation medium contains 10\(^-4\) M DNP.

The values above are means from 2 to 4 experiments which differed by no more than 10%.
Methods employed are described in sections 2.2 to 2.5, 2.8 and 2.9 of Chapter 2. Cells were preincubated as indicated below for 60 minutes. They were then transferred to either normal Krebs-Ringer medium or a modified Ringer solution wherein choline+ replaced the Na+. Both Ringers contained 1 mM glutamine, 10 mM glucose and 2 mM L-(Me-14C)methionine, specific activity 360 dpm/nmole. The uptake of methionine, cellular Na+ and ATP were estimated for 60 minutes at 25°C.

<table>
<thead>
<tr>
<th>Preincubation Conditions</th>
<th>Medium Na(^+) (μeq/ml)</th>
<th>Cellular Na(^+) (t_0) (μeq/ml cell water)</th>
<th>Cellular Na(^+) (t_{60}) (μeq/ml cell water)</th>
<th>Cellular ATP (t_0) (μoles/ml cell water)</th>
<th>Cellular ATP (t_{60}) (μoles/ml cell water)</th>
<th>L-(Me-14C)methionine Uptake (t_{60}) (nmoles/mg dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C Krebs-Ringer</td>
<td>145</td>
<td>20-30</td>
<td>35-50</td>
<td>2.5</td>
<td>3.1</td>
<td>43.0</td>
</tr>
<tr>
<td>37°C Krebs-Ringer</td>
<td>0-5</td>
<td>20-30</td>
<td>10-20</td>
<td>2.4</td>
<td>2.8</td>
<td>10.1</td>
</tr>
<tr>
<td>37°C Krebs-Ringer+DNP (b)</td>
<td>145</td>
<td>150-160</td>
<td>80-100</td>
<td>1.7</td>
<td>2.0</td>
<td>35.7</td>
</tr>
<tr>
<td>37°C Krebs-Ringer+DNP (b)</td>
<td>0-5</td>
<td>150-160</td>
<td>10-20</td>
<td>1.6</td>
<td>1.9</td>
<td>8.7</td>
</tr>
</tbody>
</table>

\(a\) A cellular concentration of 2 μmoles/ml cell water of methionine is equivalent to 7.4 nmoles/mg dry wt. in the unswollen cell.

\(b\) The medium contained 10-4 M DNP.

The values above are means from 2 or 3 experiments which differed by no more than 15%.
TABLE 5-5

PROTEIN SYNTHESIS AND THE RECOVERY OF GLYCINE TRANSPORT ACTIVITY

Methods employed are described in sections 2.2 to 2.5 of Chapter 2. Cells were preincubated for 60 minutes as indicated below, then transferred to fresh normal Krebs-Ringer medium containing 2 mM (l-14C)glycine -- specific activity 750 dpm/nmole -- and the indicated additions. The uptake of glycine and its incorporation into protein were estimated for 60 minutes at 37°C.

<table>
<thead>
<tr>
<th>Preincubation Conditions</th>
<th>Additions</th>
<th>(l-14C)glycine Uptake t60</th>
<th>(l-14C)glycine Incorporation into Protein t60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Glutamine</td>
<td>Puromycin</td>
</tr>
<tr>
<td></td>
<td>(μmoles/ml)</td>
<td>(μmoles/ml)</td>
<td>(μmoles/ml)</td>
</tr>
<tr>
<td>37°C Krebs-Ringer</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>37°C Krebs-Ringer</td>
<td>10.0</td>
<td>1.0</td>
<td>Nil</td>
</tr>
<tr>
<td>37°C Krebs-Ringer</td>
<td>10.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>37°C Krebs-Ringer+DNP a</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>37°C Krebs-Ringer+DNP a</td>
<td>10.0</td>
<td>1.0</td>
<td>Nil</td>
</tr>
<tr>
<td>37°C Krebs-Ringer+DNP a</td>
<td>10.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

a These media contained 10^-4 M DNP.

The values above are means from 2 to 4 experiments which differed by no more than 10%.
Similar results were obtained with methionine: the uptake of methionine (from media containing glucose and glutamine) was reduced only by 20% in the presence of 1 mM puromycin while its incorporation into protein was inhibited by 95%.

The results in this subsection indicate that the transport of glycine and methionine is almost entirely dependent upon the presence of cellular ATP and extracellular Na$^+$ rather than the cellular Na$^+$ concentration or the transmembrane Na$^+$ gradient. If the above is correct, then (a), in cells depleted of ATP, the Na$^+$ gradient should not cause large fluxes of amino acid, (b), amino acid uptake should show a strong dependence on the level of cellular ATP despite the presence of a "normal" inward Na$^+$ gradient and (c), cells with a "reversed" outward Na$^+$ gradient, but containing ATP, should concentrate amino acids in the presence of sufficient extracellular Na$^+$. The experiments in the next two subsections were designed to test these predictions.

5.3 MOVEMENT OF MONOVALENT CATIONS AND AMINO ACIDS IN ATP-DEPLETED CELLS

To assess the effects of monovalent cation gradients of known magnitude and direction on the movement of amino acids in ATP-depleted cells, it was first necessary to examine cation movements and gradients in those cells. Figure 5-1 has been used to illustrate that preincubation of cells in the presence of $10^{-4}$ M DNP reduces the cellular ATP level to 0.1 mM in 60 minutes. Furthermore, under these conditions, the cellular Na$^+$
concentration tends towards that of the extracellular fluid thus enabling the manipulation of the cellular Na⁺ concentration. Table 5-6 illustrates the cellular Na⁺ and K⁺ levels after a 60 minute preincubation period at 37°C in the presence of 10⁻⁴ M DNP and various extracellular concentrations of Na⁺, K⁺, and choline⁺. When ATP-depleted cells containing high and low levels of Na⁺ and almost no K⁺ (see Table 5-6) are then placed in, (a), normal Krebs-Ringer medium or (b), modified Ringer medium wherein the Na⁺ has been totally replaced with choline⁺ and both containing 10⁻⁴ M DNP to maintain the ATP-depleted state, four basic Na⁺ gradient conditions should be created: (a), a "normal" inward Na⁺ gradient, (b), a "reversed" outward Na⁺ gradient, and essentially no Na⁺ gradient when either, (c), high or (d), low levels of Na⁺ are placed on both sides of the membrane. Figure 5-2A contains the results of a typical experiment illustrating the changes in cellular Na⁺ concentration when ATP-depleted cells containing 30 and 170 mM Na⁺ (and 0-25 mM K⁺) are each treated as described above. Figure 5-2B contains the same data replotted to illustrate the Na⁺ gradients thus created. It is apparent that the prescribed manipulations were successful in creating the predicted "normal" and "reversed" Na⁺ gradients. However, when large or small concentrations of Na⁺ were placed on both sides of the membrane, an outward gradient of small magnitude was observed rather than no gradient at all.

It should also be possible to create gradient conditions similar to those described above for Na⁺, but using Na⁺ and K⁺.
### TABLE 5-6

**VARIATION OF CELLULAR Na⁺ AND K⁺ CONCENTRATIONS DUE TO CHANGES IN THE COMPOSITION OF DNP-CONTAINING MEDIA**

Methods employed are described in sections 2.2 to 2.5 and 2.8 of Chapter 2. Cells were incubated at 37°C for 60 minutes in normal Krebs-Ringer medium or Ringer solutions modified as indicated below. All media contained 10⁻⁴ M DNP.

<table>
<thead>
<tr>
<th>Extracellular</th>
<th>Cellular</th>
<th>Choline⁺</th>
<th>Na⁺ (μeq/ml cell water)</th>
<th>K⁺ (μeq/ml cell water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺ (μeq/ml)</td>
<td>K⁺ (μeq/ml)</td>
<td></td>
<td>172 ± 9</td>
<td>24 ± 12</td>
</tr>
<tr>
<td>145</td>
<td>8</td>
<td>Nil</td>
<td>41 ± 3</td>
<td>170 ± 4</td>
</tr>
<tr>
<td>40</td>
<td>113</td>
<td>Nil</td>
<td>26*</td>
<td>190*</td>
</tr>
<tr>
<td>20</td>
<td>133</td>
<td>Nil</td>
<td>20*</td>
<td>175*</td>
</tr>
<tr>
<td>Nil</td>
<td>153</td>
<td>Nil</td>
<td>25 ± 5</td>
<td>0-5</td>
</tr>
<tr>
<td>Nil</td>
<td>8</td>
<td>145</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* One observation only.

The values above are means ± S.D. from 2 to 5 experiments.
Figures 5-2A and 5-2B. Cell Na\(^+\) movements and the Na\(^+\) gradient in ATP depleted cells. Methods employed are described in sections 2.2, 2.3, and 2.8 of Chapter 2 as well as 5.3 of this Chapter. Cells initially loaded with Na\(^+\) were placed in normal Krebs-Ringer medium (▲) or a modified Ringer medium wherein choline\(^+\) replaced the Na\(^+\) (△). Similarly, cells initially containing little Na\(^+\) were placed in normal (●) or modified (○) Ringer. All media contained 10\(^{-4}\) M DNP. Figure 5-2A depicts the changes in cellular Na\(^+\) at 25°C while Figure 5-2B illustrates the corresponding Na\(^+\) gradients.
However, the gradient situations for $K^+$ would necessarily be the converse of those for $Na^+$. The four basic gradient conditions in this case should be: (a), a "normal" inward $Na^+$ plus outward $K^+$ gradient, (b), a "reversed" outward $Na^+$ plus inward $K^+$ gradient, essentially no $Na^+$ or $K^+$ gradients with either (c), high levels of $Na^+$ and low levels of $K^+$ or (d), low levels of $Na^+$ and high levels of $K^+$ on both sides of the membrane. Figures 5-3A and 5-3B show the changes in cellular $Na^+$ and $K^+$ respectively when ATP-depleted cells containing approximately 30 mM $Na^+$ and 175 mM $K^+$ are transferred to, (a), normal Krebs-Ringer medium or (b), modified Ringer medium wherein $K^+$ has totally replaced the $Na^+$ and both containing $10^{-4}$ M DNP. Figures 5-4A and 5-4B illustrate the same for ATP-depleted cells containing approximately 180 mM $Na^+$ and 15 mM $K^+$. It is apparent that when the cellular and extracellular levels of $Na^+$ and $K^+$ differ initially, these cations will move so that their cellular concentrations tend to approach those of the extracellular fluid. When the data in Figures 5-3A, 5-3B, 5-4A and 5-4B were recalculated to illustrate the $Na^+$ (Table 5-7) and $K^+$ (Table 5-8) gradients thus created, it was apparent that the predicted "normal" and "reversed" gradients were both present. However, the small outward cation gradients were again observed under conditions where no gradients were predicted. Furthermore, all artificially created gradients described above were largely dissipated within 30 minutes.
FIGURES 5-3A AND 5-3B. CELL Na⁺ AND K⁺ MOVEMENTS IN K⁺-LOADED, ATP-DEPLETED CELLS. Methods employed are described in sections 2.2, 2.3 and 2.8 of Chapter 2 as well as 5.3 of this Chapter. Cells initially containing 20-40 µeq of Na⁺ and 165-175 µeq of K⁺ per ml cell water were placed in normal Krebs-Ringer medium (○) and a modified Ringer medium wherein K⁺ replaced the Na⁺ (●). All media contained 10⁻⁴ M DNP. Figure 5-3A depicts the changes in cell Na⁺ at 25°C while the changes in cell K⁺ are illustrated in Figure 5-3B.
FIGURES 5-4A AND 5-4B. CELL Na⁺ AND K⁺ MOVEMENTS IN Na⁺-LOADED, ATP-DEPLETED CELLS. Methods employed are described in sections 2.2, 2.3 and 2.8 of Chapter 2 and 5.3 of this Chapter. Cells initially containing 170-190 μeq of Na⁺ and 8-15 μeq of K⁺ per ml cell water were placed in normal Krebs-Ringer medium (A,○; B,●) and a modified Ringer medium wherein K⁺ replaced the Na⁺ (A,●; B,○). All media contained 10⁻⁴ M DNP. Figure 5-4A illustrates the changes in cell Na⁺ at 25°C while changes in cell K⁺ are shown in Figure 5-4B.
## TABLE 5-7

**ARTIFICIALLY CREATED Na⁺ GRADIENTS IN ATP-DEPLETED CELLS**

Methods and conditions are described in the Legends of Figures 5-3 and 5-4.

<table>
<thead>
<tr>
<th>Initial Cellular Na⁺ K⁺ (μeq/ml cell water)</th>
<th>Extracellular Na⁺ K⁺ (μeq/ml)</th>
<th>t₀</th>
<th>t₂</th>
<th>t₅</th>
<th>t₁₀</th>
<th>t₃₀</th>
<th>t₆₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-42 168-176</td>
<td>145 8</td>
<td>a</td>
<td>b</td>
<td>a</td>
<td>b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>166-190 8-24</td>
<td>145 8</td>
<td>20-45 10-45 --- 30-60 0-55 10-20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>166-190 8-24</td>
<td>Nil 153</td>
<td>165-190 90-135 100-130 60-130 50-110 35-95</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- a Inward Na⁺ gradient: the extracellular Na⁺ concentration > the cellular Na⁺ concentration.
- b Outward Na⁺ gradient: the extracellular Na⁺ concentration < the cellular Na⁺ concentration.
### TABLE 5-8

**ARTIFICIALLY CREATED K⁺ GRADIENTS IN ATP-DEPLETED CELLS**

Methods and conditions are described in the Legends of Figures 5-3 and 5-4.

<table>
<thead>
<tr>
<th>Initial Cellular Na⁺ K⁺ (μeq/ml cell water)</th>
<th>Extracellular Na⁺ K⁺ (μeq/ml)</th>
<th>Potassium Gradient Range at ( t_0 ) (μeq/ml)</th>
<th>Potassium Gradient Range at ( t_2 ) (μeq/ml)</th>
<th>Potassium Gradient Range at ( t_5 ) (μeq/ml)</th>
<th>Potassium Gradient Range at ( t_{10} ) (μeq/ml)</th>
<th>Potassium Gradient Range at ( t_{30} ) (μeq/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-42</td>
<td>168-176</td>
<td>145 8</td>
<td>a 160-170</td>
<td>95-150</td>
<td>70-135</td>
<td>40-110</td>
</tr>
<tr>
<td>17-42</td>
<td>168-176</td>
<td>Nil 153</td>
<td>a 15-25</td>
<td>--</td>
<td>--</td>
<td>15-35</td>
</tr>
<tr>
<td>166-190</td>
<td>8-24</td>
<td>145 8</td>
<td>a 15-25</td>
<td>--</td>
<td>5-10</td>
<td>5-10</td>
</tr>
<tr>
<td>166-190</td>
<td>8-24</td>
<td>Nil 153</td>
<td>b 130-145</td>
<td>60-105</td>
<td>55-90</td>
<td>50-80</td>
</tr>
</tbody>
</table>

a  **Outward K⁺ gradient:** the extracellular K⁺ concentration < the cellular K⁺ concentration.

b  **Inward K⁺ gradient:** the extracellular K⁺ concentration > the cellular K⁺ concentration.
In conclusion, the manipulation of cellular (and extracellular) cation concentrations is possible in the ATP-depleted cell. Estimation of the cellular cation levels enabled the calculation of the magnitude, direction and rate of decay of the cation gradients artificially created via such manipulation. The influence of these gradients on the movement of amino acids in ATP-depleted cells and the gradient-induced movement of amino acids in ATP-depleted cells could then be assessed.

To determine the effect of the Na$^+$ gradient on the uptake and efflux of methionine in ATP-depleted cells, preincubations were performed to obtain ATP-depleted cells containing 30 and 170 mM Na$^+$ and very little K$^+$ (see Table 5-6). These cells were subsequently transferred into, (a), normal Krebs-Ringer medium or (b), modified Ringer medium wherein choline$^+$ totally replaced Na$^+$, both containing $10^{-4}$ M DNP. The changes in cellular Na$^+$ and the Na$^+$ gradient were similar to those shown in Figures 5-2A and 5-2B respectively. To measure efflux, cells were loaded with approximately 4 mM L-(Me-$^{14}$C)methionine during the preincubation period, transferred to amino acid-free media, and the loss of cellular radioactivity estimated with time. To measure uptake, cells were preincubated in amino acid-free media, incubated in the presence of 2 mM L-(Me-$^{14}$C)methionine, and the appearance of cellular radioactivity estimated with time. The results are illustrated in Figures 5-5A and 5-5B. It is apparent that while the Na$^+$ gradient had no effect on the efflux of
FIGURES 5-5A AND 5-5B. THE Na⁺ GRADIENT AND METHIONINE EFFLUX AND UPTAKE IN ATP-DEPLETED CELLS AT 25°C. Methods employed are described in sections 2.2 to 2.5 of Chapter 2 and 5.3 of this Chapter. Cells initially containing 30 µeq of Na⁺/ml cell water were placed in normal Krebs-Ringer medium (●) or a modified Ringer medium wherein choline⁺ replaced the Na⁺ (○). Similarly, cells initially containing 170 µeq of Na⁺/ml cell water were placed in normal (▲) or modified (△) Ringers. All media contained 10⁻⁴ M DNP. In Figure 5-5A, cells were initially loaded with approximately 4 µmoles of L-(Me-¹⁴C)methionine/ml cell water while in Figure 5-5B, 2 mM L-(Me-¹⁴C)methionine was placed in the medium. (Specific activities were 360 dpm/nmole in each case.)
methionine, the efflux was least when there was little Na\(^+\) on both sides of the membrane. Methionine uptake, however, is very sensitive to the presence of extracellular Na\(^+\); the cellular methionine concentration reaches that of the medium only in the presence of extracellular Na\(^+\). Furthermore, high levels of Na\(^+\) on both sides of the membrane or a "reversed" outward Na\(^+\) gradient (in the presence of little extracellular Na\(^+\)) both result in only a small reduction of the observed uptakes. It was therefore concluded that there is little or no effect of Na\(^+\) or its gradients on methionine efflux but the uptake of methionine into ATP-depleted cells is sensitive to extracellular Na\(^+\) and much more so than to the Na\(^+\) gradient.

Assessing the effects of the Na\(^+\) gradient on amino acid movements in ATP-depleted cells is complicated by the tendency of the amino acid to move down its own concentration gradient (Figures 5-5A, 5-5B). To avoid this problem, the cation gradient(s) can be imposed subsequent to placing equal concentrations of amino acid on both sides of the membrane. It is then possible to estimate amino acid movements induced by the cation gradient(s). The following is a description of the applied procedure. Cells were preincubated as outlined in Table 5-6 for 30 to 45 minutes to deplete cellular ATP and adjust the cellular ionic composition. Then, either \(^{14}\)C-amino acid or \(^{14}\)C-thiourea (a non-actively transported compound) was added to the medium and the pretreatment continued for a further 60 minutes when the cellular
concentration of the radioactive material was estimated (see section 2.4 of Chapter 2). The cells were subsequently introduced into fresh media containing $10^{-4}$ M DNP and the radioactive material at the same concentration and specific activity as that found intracellularly. The ionic composition of these fresh media were varied to obtain the ion gradients shown in Tables 5-7 and 5-8. Thus any change in cellular radioactivity constituted a net movement against a concentration gradient unless compensated by changes in cell fluid. Cell water shifts were determined by weighing each cell sample (see section 2.5 of Chapter 2).

Since it was of interest to determine whether or not gradients of either $\text{Na}^+$ or $\text{K}^+$ could separately stimulate net amino acid movement, cells were depleted of ATP and pre-equilibrated with $\text{L-}(\text{Me}^{-14}\text{C})$methionine as above. Gradients were created as in Tables 5-7 and 5-8 except that in one batch of cells choline$^+$ replaced the cellular and extracellular $\text{K}^+$, while in a second batch it was used to replace cellular and extracellular $\text{Na}^+$. The ion gradient-induced movement of $\text{L-}(\text{Me}^{-14}\text{C})$methionine against its own concentration gradient was estimated and appears in Table 5-9. The results indicate that gradients of this type could not stimulate any sizable uptake of methionine against its own concentration gradient. However, both "normal" inward $\text{Na}^+$ and outward $\text{K}^+$ gradients of initially large magnitude allowed the least loss, while both "reversed" outward $\text{Na}^+$ and inward $\text{K}^+$ gradients
TABLE 5-9

Na⁺ GRADIENT AND K⁺ GRADIENT INDUCED FLUX OF METHIONINE AND
THIOUREA IN ATP-DEPLETED CELLS

Methods employed are described in sections 2.2 to 2.5 and 2.8 of Chapter 2 and section 5.3 of this
Chapter. Cells were depleted of ATP and pre-equilibrated with 3 mM concentrations of L-(Me-¹⁴C)-
methionine or (¹⁴C)thiourea -- specific activities were 360 dpm/nmole. The ion gradients indicated
below were created artificially. The incubation media contained 10⁻⁴ M DNP and 3 mM concentrations
of L-(Me-¹⁴C)methionine or (¹⁴C)thiourea of the same specific activity as that above. Cellular
radioactivity was estimated for 30 minutes at 25°C.

<table>
<thead>
<tr>
<th>Extracellular Cation Concentration (μeq/ml)</th>
<th>Initial Cellular Concentration (μeq/ml cell water)</th>
<th>Cation Gradient Direction</th>
<th>Cation Gradient Magnitude (μeq/ml) t₂</th>
<th>t₃₀</th>
<th>2 to 30 min. Uptake of L-(Me-¹⁴C)meth. (¹⁴C)thiourea (μmoles/ml cell water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>145</td>
<td>20-30</td>
<td>inward</td>
<td>80-120</td>
<td>10-60</td>
<td>0.10 a</td>
</tr>
<tr>
<td>145</td>
<td>160-180</td>
<td>outward</td>
<td>10-40</td>
<td>0-30</td>
<td>-0.10</td>
</tr>
<tr>
<td>Nil</td>
<td>20-30</td>
<td>outward</td>
<td>10-30</td>
<td>10-20</td>
<td>-0.42</td>
</tr>
<tr>
<td>Nil</td>
<td>160-180</td>
<td>outward</td>
<td>140-180</td>
<td>50-110</td>
<td>-0.75</td>
</tr>
<tr>
<td>K⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>165-175</td>
<td>outward</td>
<td>100-150</td>
<td>30-80</td>
<td>-0.02</td>
</tr>
<tr>
<td>153</td>
<td>165-175</td>
<td>outward</td>
<td>15-25</td>
<td>0-10</td>
<td>-0.27</td>
</tr>
<tr>
<td>8</td>
<td>5-25</td>
<td>outward</td>
<td>0-15</td>
<td>0-10</td>
<td>-0.50</td>
</tr>
<tr>
<td>153</td>
<td>5-25</td>
<td>inward</td>
<td>60-100</td>
<td>40-70</td>
<td>-0.66</td>
</tr>
</tbody>
</table>

a A minus sign denotes efflux.

The values above are means from 3 to 5 experiments which differed by no more than 20%.
of initially large magnitude induced the greatest loss of cellular methionine.

Although the Na\(^+\) or K\(^+\) gradient could not separately induce amino acid uptake against its own concentration gradient, it was possible that the combined effects of the Na\(^+\) plus K\(^+\) gradients could do so. Cells were therefore depleted of ATP and pre-equilibrated with (L-\(^{14}\)C)glycine, L-(Me-\(^{14}\)C)methionine, L-(L-\(^{14}\)C)-leucine or \((^{14}\)C)thiourea. Gradients were created as in Tables 5-7 and 5-8. The cation gradient-induced movements of the aforementioned radioactive compounds against their own concentration gradients were estimated and appear in Table 5-10. The results show that, (a), the "normal" inward Na\(^+\) plus outward K\(^+\) gradients of large magnitude induced the most uptake while, (b), the "reversed" outward Na\(^+\) plus inward K\(^+\) gradients induced the most efflux of all four compounds. Furthermore, when there was little Na\(^+\) or K\(^+\) gradient, some uptake of glycine and methionine was observed when high levels of Na\(^+\) were placed on both sides of the membrane, while some efflux was detected when high levels of K\(^+\) were placed intra- and extracellularly. It was therefore concluded that in ATP-depleted cells, the Na\(^+\)-K\(^+\) gradient system could induce a modest amount of net amino acid flux against its own concentration gradient.

The evidence presented in this subsection can be summarized as follows. In ATP-depleted Ehrlich ascites cells, four basic monovalent cation gradient situations can be created artificially.
TABLE 5-10

THE Na⁺ PLUS K⁺ GRADIENT-INDUCED FLUX OF METHIONINE, GLYCINE, LEUCINE AND THIOUREA IN ATP-DEPLETED CELLS

Methods employed are described in sections 2.2 to 2.5 and 2.8 of Chapter 2 and section 5.3 of this Chapter. Cells were depleted of ATP and pre-equilibrated with 3 mM concentrations of L-((Me-14C))-methionine, (14C)glycine, L-((1-14C)leucine or (14C)thiourea -- specific activities were 360 dpm/nmole. Cells were then transferred to the incubation media which contained 10⁻⁴ M DNP and 3 mM concentrations of the labeled compounds above at the same specific activities as above. Cellular radioactivity was estimated for 30 minutes at 25°C.

<table>
<thead>
<tr>
<th>Extracellular Na⁺ K⁺</th>
<th>Cellular Na⁺</th>
<th>Cellular K⁺</th>
<th>2 to 30 minute net uptakes of</th>
<th>a</th>
</tr>
</thead>
<tbody>
<tr>
<td>(μeq/ml)</td>
<td>(μeq/ml cell water)</td>
<td>(μeq/ml cell water)</td>
<td>methionine</td>
<td>glycine</td>
</tr>
<tr>
<td>145 8</td>
<td>30-60</td>
<td>90-135</td>
<td>105-165</td>
<td>30-85</td>
</tr>
<tr>
<td>145 8</td>
<td>170-210</td>
<td>160-200</td>
<td>10-15</td>
<td>10-15</td>
</tr>
<tr>
<td>Nil 153</td>
<td>10-25</td>
<td>5-15</td>
<td>165-185</td>
<td>150-165</td>
</tr>
<tr>
<td>Nil 153</td>
<td>90-135</td>
<td>50-110</td>
<td>80-95</td>
<td>85-120</td>
</tr>
</tbody>
</table>

⁻ Minus signs denote efflux.

The values above are means from 2 to 5 experiments which differed by no more than 20%.
Two of these, the "normal" and "reversed" Na\(^+\) plus K\(^+\) gradients, were found to induce a modest amount of net uphill amino acid flux. Furthermore, neither the "normal" inward Na\(^+\) nor "normal" outward K\(^+\) gradient could separately induce any net influx, but could reduce the net loss of amino acid.

5.4 AMINO ACID MOVEMENT IN ATP-CONTAINING AND ATP-DEPLETED CELLS

The results in Tables 5-7 and 5-8 indicate that in ATP-depleted cells, the artificially created cation gradients were subject to continuous dissipation over the first 30 minutes of incubation. Therefore, the maximum amino acid flux induced by a Na\(^+\) plus K\(^+\) gradient of a given magnitude cannot be estimated and subsequently compared to the uptake in ATP containing cells. However, the results in Tables 5-7 and 5-8 also show that between 2 and 5 minutes, the "normal" inward Na\(^+\) and outward K\(^+\) gradients in the ATP-depleted cell are of a magnitude comparable to those of the ATP containing cell (Na\(^+\): 95-105 mM, K\(^+\): 145-155 mM). Comparison of the net amino acid uptakes over the period of time when the cation gradients are similar in both ATP-depleted and ATP-containing cells should provide some further indication whether uphill amino acid transport is primarily dependent upon cellular ATP or the transmembrane Na\(^+\) plus K\(^+\) gradients. Cells were therefore depleted of ATP in a modified Ringer solution wherein K\(^+\) replaced the Na\(^+\) (Table 5-6). They were then loaded with (1-\(^{14}\)C)glycine, L-(Me-\(^{14}\)C)methionine,
L-(1-\textsuperscript{14}C)leucine or \textsuperscript{(14}C)thiourea. Cells containing ATP were also loaded with the above compounds. Both types of cell were subsequently transferred to normal Krebs-Ringer medium containing the aforementioned compounds at concentrations and specific activities equal to those in the cell fluid. The medium into which the ATP-depleted cells were placed also contained $10^{-4}$ M DNP. The net uptake of (1-\textsuperscript{14}C)glycine, L-(Me-\textsuperscript{14}C)methionine, L-(1-\textsuperscript{14}C)leucine and \textsuperscript{(14}C)thiourea against their own concentration gradients was estimated between 2 and 5 minutes when the magnitude of the normal Na\textsuperscript{+} and K\textsuperscript{+} gradients in both ATP-depleted and ATP-containing cells are comparable. Table 5-11 shows that in cells with Na\textsuperscript{+} and K\textsuperscript{+} gradients of comparable magnitude, the net uptake of (1-\textsuperscript{14}C)glycine, L-(Me-\textsuperscript{14}C)methionine, and L-(1-\textsuperscript{14}C)leucine is considerably smaller in ATP-depleted than ATP-containing cells. These results are consistent with the conclusion that the uptake of amino acids is primarily dependent upon cellular ATP rather than the transmembrane Na\textsuperscript{+}-K\textsuperscript{+} gradient system.

Cells in which amino acid transport is primarily dependent upon ATP and extracellular Na\textsuperscript{+} should concentrate amino acids in the presence of sufficient extracellular Na\textsuperscript{+} and ATP even though there is a reversed Na\textsuperscript{+} plus K\textsuperscript{+} gradient across the plasma membrane. To test this prediction, cells were preincubated at 10\textdegree C in normal Krebs-Ringer medium to contain 1.0 to 2.0 mM ATP and high levels of Na\textsuperscript{+} (Table 3-1). For comparison, Na\textsuperscript{+}-loaded,
TABLE 5-11

THE EFFECT OF ATP ON THE UPTAKE OF GLYCINE, METHIONINE, LEUCINE AND THIOUREA INTO CELLS WITH NORMAL Na⁺ PLUS K⁺ GRADIENTS

Methods employed are described in sections 2.2 to 2.5 and 2.8 of Chapter 2 and section 5.3 of this Chapter. ATP-depleted and ATP-containing cells were pre-equilibrated with 3 mM concentrations of (1-14C)glycine, L-(Me-14C)methionine, L-(1-14C)leucine or (14C)thiourea -- specific activities 360 dpm/n mole. Cells were then transferred to incubation media which contained 3 mM concentrations of those compounds at the same specific activities as above. Cellular radioactivity was estimated for 5 minutes at 25°C.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Inward Na⁺ Gradient (μeq/ml)</th>
<th>Outward K⁺ Gradient (μeq/ml)</th>
<th>Extracellular 2,4-DNP (μmoles/ml)</th>
<th>Net Uptake between 2 and 5 min. (nmoles/mg dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1-14C)glycine</td>
<td>95-105</td>
<td>145-155</td>
<td>Nil</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>70-100</td>
<td>85-145</td>
<td>0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>L-(Me-14C)methionine</td>
<td>95-105</td>
<td>145-155</td>
<td>Nil</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>70-100</td>
<td>85-145</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>L-(1-14C)leucine</td>
<td>95-105</td>
<td>145-155</td>
<td>Nil</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>70-100</td>
<td>85-145</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>(14C)thiourea</td>
<td>95-105</td>
<td>145-155</td>
<td>Nil</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>70-100</td>
<td>85-145</td>
<td>0.1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The values above are means from 2 to 5 experiments which differed by no more than 20%.
ATP-depleted cells were also prepared (Table 5-6). Both types of cell were then loaded with L-(Me-\(^{14}\)C)methionine and subsequently transferred to modified Ringer media wherein, (a), 125 mM Na\(^+\) and (b), 65 mM Na\(^+\) had been replaced with K\(^+\). Both solutions contained L-(Me-\(^{14}\)C)methionine of equal concentration and specific activity to that in the cells. The media which received the ATP-depleted cells also contained \(10^{-4}\) M DNP. The net flux of L-(Me-\(^{14}\)C)methionine against its concentration gradient under these conditions is illustrated in Figure 5-6. It is apparent that in media containing only 20 mM Na\(^+\), the Na\(^+\) loaded cells lost amino acid whether or not they contained ATP. However, in the presence of 80 mM extracellular Na\(^+\), the ATP-containing cells concentrated methionine while the ATP-depleted cells did not gain or lose any methionine. It should be emphasized that in the presence of 80 mM Na\(^+\) extracellularly, the magnitude of the "reversed" Na\(^+\) gradient was initially 80 to 100 mM, while that in the presence of 20 mM Na\(^+\) extracellularly was initially 130 to 160 mM. These results corroborate the original conclusion (section 5.2) that amino acid transport in Ehrlich ascites cells is primarily dependent on cellular ATP and extracellular Na\(^+\) rather than the transmembrane monovalent cation gradients.

5.5 THE EFFECT OF Na\(^+\) AND ATP ON NET AMINO ACID UPTAKE

The results in Chapter 4 indicate that the initial rate of net amino acid uptake into ATP-containing cells was stimulated
FIGURE 5-6. THE EFFECTS OF ATP AND THE REVERSED Na⁺ GRADIENT ON METHIONINE MOVEMENT AT 25°C. Methods employed are described in sections 2.2 to 2.5 of Chapter 2 as well as 5.3 and 5.4 of this Chapter. Na⁺-loaded, ATP-containing cells were placed in modified media containing 20 mM Na⁺ (○) or 80 mM Na⁺ (●). Similarly, Na⁺-loaded ATP-depleted cells were incubated in the 20 mM Na⁺ (△) or 80 mM Na⁺ (▲) containing media, but in the presence of 10⁻⁴ M DNP. Both the cells and the media initially contained 5 mM L-(Me-¹⁴C)methionine, specific activity, 450 dpm/nmole.
by high levels of extracellular Na\(^+\). The results in this Chap-
ter have shown that while cellular Na\(^+\) and the Na\(^+\) gradient
have only marginal effects on net amino acid uptake, ATP greatly
stimulates the initial rate of net amino acid transport. It was
of interest to determine whether or not ATP and extracellular
Na\(^+\) exert their stimulatory effects in an independent manner.
Thus, the initial rates of net uptake of (l-\(^{14}\text{C})\text{glycine and L-}
(Me-\(^{14}\text{C})\text{ methionine were measured as a function of the extracel-
lar concentrations of those amino acids and Na}\(^+\) in ATP-
containing and ATP-depleted cells. Cells were pretreated for
60 minutes at 37\(^\circ\)C in either normal Krebs-Ringer medium or a
modified Ringer solution wherein K\(^+\) replaced the Na\(^+\) which also
contained 10\(^{-4}\) M DNP. Though only half the cells contained ATP,
all cells contained 20-30 mM Na\(^+\) and 150-180 mM K\(^+\) (Tables 5-2
and 5-6). The cells were subsequently introduced into a modi-
fied Ringer medium containing various concentrations of Na\(^+\),
choline\(^+\), and \(^{14}\text{C}-\text{amino acid. The media into which the ATP-
depleted cells were placed also contained 10}^{-4}\text{ M DNP. The ini-
tial rate of net (l-\(^{14}\text{C})\text{glycine uptake was estimated under the
conditions above. When measuring L- (Me-}\(^{14}\text{C})\text{methionine uptake,
cells preincubated in the presence of 0.1 mM L- (Me-}\(^{14}\text{C})\text{methio-
nine were used so that the endogenous exchangeable free amino
acid pool (section 4.2 of Chapter 4) was completely replaced by
radioactive methionine. The initial increase in cellular radio-
activity due to the presence of various concentrations of extra-
cellular L- (Me-}\(^{14}\text{C})\text{methionine was considered to be net uptake.}
FIGURE 5-7. EFFECT OF Na\(^+\) AND ATP ON THE UPTAKE OF GLYCINE AT 25°C. Methods employed are described in sections 2.2 to 2.5 of Chapter 2 and 5.5 of this Chapter. All cells initially contained 20-30 µeq of Na\(^+\) and 150-180 µeq of K\(^+\) per ml cell water. Open symbols represent cells depleted of ATP and incubated in the presence of 10^{-4} M DNP while closed symbols represent ATP-containing cells. The (L-\(^{14}\)C)-glycine used had a specific activity of 750 dpm/nmole.
FIGURE 5-8. EFFECT OF Na$^+$ AND ATP ON THE UPTAKE OF METHIONINE AT 25°C. Methods employed are described in sections 2.2 to 2.5 of Chapter 2 and 5.5 of this Chapter. All cells initially contained 20-30 μeq of Na$^+$ and 150-180 μeq of K$^+$ per ml cell water. Open symbols represent cells depleted of ATP and incubated in the presence of 10$^{-4}$ M DNP while closed symbols represent ATP-containing cells. The L-(Me-$^{14}$C)methionine used had a specific activity of 750 dpm/nmole.
### Table 5.12

**The Effect of ATP and Extracellular Na\(^+\) on the Kinetic Parameters for Methionine and Glycine Uptake**

Methods and conditions are described in the Legends of Figures 5-9 and 5-10.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Extracellular Na(^+) (µg/ml)</th>
<th>L-(Me-(^{14})C)methionine</th>
<th>(1-(^{14})C)glycine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K(_m) (µmoles/ml)</td>
<td>V(_{max}) (nmoles/10 mg dry wt)</td>
<td>K(_m) (µmoles/ml)</td>
</tr>
<tr>
<td>ATP-containing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>-</td>
<td>0.4</td>
<td>1.6</td>
</tr>
<tr>
<td>75</td>
<td>-</td>
<td>0.4</td>
<td>2.6</td>
</tr>
<tr>
<td>50</td>
<td>0.7</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>-</td>
<td>95</td>
<td>4.4</td>
</tr>
<tr>
<td>25</td>
<td>1.7</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>6.3</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>ATP-depleted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>-</td>
<td>1.7</td>
<td>6.3</td>
</tr>
<tr>
<td>75</td>
<td>1.7</td>
<td>95</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>2.5</td>
<td>8.0</td>
</tr>
<tr>
<td>40</td>
<td>-</td>
<td>95</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>2.5</td>
<td>95</td>
<td>11.0</td>
</tr>
<tr>
<td>0</td>
<td>6.3</td>
<td>95</td>
<td>-</td>
</tr>
</tbody>
</table>
**TABLE 5-13**

THE EFFECT OF ATP ON THE Na⁺ REQUIREMENT FOR GLYCINE AND METHIONINE UPTAKE

Methods and conditions are described in the Legends of Figures 5-9 and 5-10.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Amino Acid</th>
<th>Na⁺ required for half maximal uptake (μeq/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP-containing</td>
<td>( \text{(L}^{14}\text{C)glycine} - 1 \text{μmole/ml} )</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>( - 2 \text{μmole/ml} )</td>
<td>31</td>
</tr>
<tr>
<td>ATP-depleted</td>
<td>( - 1 \text{μmole/ml} )</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>( - 2 \text{μmole/ml} )</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>( - 4 \text{μmole/ml} )</td>
<td>31</td>
</tr>
<tr>
<td>ATP-containing</td>
<td>( \text{L-(Me}^{14}\text{C)methionine} - 1 \text{μmole/ml} )</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>( - 2 \text{μmole/ml} )</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>( - 4 \text{μmole/ml} )</td>
<td>13</td>
</tr>
<tr>
<td>ATP-depleted</td>
<td>( - 2 \text{μmole/ml} )</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>( - 4 \text{μmole/ml} )</td>
<td>13</td>
</tr>
</tbody>
</table>
The results in Figures 5-7 and 5-8 show that ATP and extracellular Na⁺ decrease the apparent Michaelis constant for both glycine and methionine uptake leaving the maximum uptakes essentially unchanged. The apparent kinetic parameters relevant to Figures 5-7 and 5-8 are listed in Table 5-12. The ¹⁴C-amino acid uptakes (some of which are shown in Figures 5-7 and 5-8) were replotted as a function of the extracellular Na⁺ concentration according to the method of Lineweaver and Burk (117). The results indicated that the maximum uptake obtainable at infinite extracellular Na⁺ concentration was elevated in the presence of ATP but the extracellular Na⁺ concentration required to achieve half maximal glycine or methionine uptake is unchanged in the presence or absence of ATP (Table 5-13). From the results in this subsection, it was concluded that both ATP and extracellular Na⁺ independently increase the association between the amino acid carrier and its substrate.

5.6 SUMMARY

The cellular concentrations of Na⁺, K⁺, and ATP could be varied independently and monovalent cation gradients could be artificially created.

The net transport of several neutral amino acids was better correlated to the level of ATP and extracellular Na⁺ rather than to the magnitude of the transmembrane Na⁺ plus K⁺ gradient system. ATP and extracellular Na⁺ independently stimulate the net
transport of glycine and methionine by increasing the association between carrier and substrate. This evidence indicates that the Na\(^+\) gradient hypothesis (99,103) is not applicable to the transport of neutral amino acids in Ehrlich ascites cells.
6.1 NET AMINO ACID TRANSPORT

The concept of amino acid transport driven by the downhill movement of Na\(^+\) and/or K\(^+\) across the plasma membrane was first suggested by Riggs et al (99) in an effort to explain (a), the net loss of cellular K\(^+\) from Ehrlich cells accompanying the net accumulation of several amino acids and (b), the subnormal amino acid uptake resulting from depressed levels of cellular K\(^+\). Of the two cation fluxes in question, these authors considered that of K\(^+\) to be the predominant influence. They also viewed the necessity for small concentrations of extracellular K\(^+\) to be a reflection of the requirement for high levels of cellular K\(^+\) and an outward K\(^+\) gradient.

Crane (103), studying sugar transport in small intestine, developed the proposal of Riggs et al (stated above) into what is presently known as the Na\(^+\) gradient hypothesis. The evidence which prompted the hypothesis consisted mainly of two observations: (a), extracellular Na\(^+\) strongly increased the affinity of the carrier for the sugar and (b), in the absence of extracellular Na\(^+\), extracellular K\(^+\) decreased that affinity (103). Similar relationships were ascribed to these cations at the inner surface of the membrane without the benefit of experimental
verification until the latter was in part supplied by Hajjar et al (107) some years later. These authors demonstrated that cellular Na\(^+\) increased the affinity between carrier and transport substrate. Thus, according to the hypothesis, the Na\(^+\) and K\(^+\) gradients and fluxes across the membrane would be responsible for creating the asymmetry of affinities which results in the accumulation of transport substrates.

The importance of the Na\(^+\) gradient hypothesis is that it has "... transferred the problem of active transport of these amino acids and sugars into the province of facilitated diffusion resulting in a dramatic simplification of the conceptual framework in which these problems have been studied" (233). Because of the potential applicability of the hypothesis to Na\(^+\)-sensitive organic substrate transport in practically all mammalian tissues, a series of experimental tests have been proposed to examine its validity (233). These tests serve as guides to the examination of the relationship between the direction of cation and organic substrate movements across the membrane. Unfortunately the tests do not stress comparisons between, (a), gradient induced transport in the absence of metabolic energy and (b), transport in the presence of cation gradients as well as metabolic energy in the form of ATP. They have, however, provided a means for the coordination of research into the problem which has yielded the following information. First, several investigators have indicated either by calculation and/or
experimental demonstration that there is sufficient energy in the transmembrane Na\(^+\) gradient or Na\(^+\) plus K\(^+\) gradients to drive the accumulation of organic compounds observed experimentally (102,103,106,193). Second, the cation gradients can cause net movement of amino acids (90,100,102,105-107). Third, cation gradients of suitable orientation can drive net amino acid transport into and out of the cell (100,105-107) while gradients of amino acids can also cause the net movement of Na\(^+\) (108,174) and K\(^+\) (174).

The evidence above clearly supports the view that the Na\(^+\) gradient hypothesis is applicable to Na\(^+\)-sensitive amino acid transport and that many of the observed effects characteristic of that transport can be explained in terms of the hypothesis. Accordingly, any chemical agent (metabolic inhibitor, cardiac glycoside, SH-binding compounds, etc.) or environmental manipulation (removal and replacement of cellular and extracellular Na\(^+\) and K\(^+\)) which, when imposed on the system, results in a change in the transmembrane distribution of Na\(^+\) and K\(^+\) will indirectly affect the net transport of amino acids. For example, poisoning cells with cyanide or DNP limits the availability of metabolic energy which results in a decrease in the magnitude of the asymmetric transmembrane distribution of Na\(^+\) and K\(^+\). This, in turn, reduces the electrochemical potential energy available from the cation gradients that drive the net transport of amino acids, thus limiting that transport.
During the course of the aforementioned investigations concerning the Na\(^+\) gradient hypothesis, several observations were reported which were strikingly inconsistent with the hypothesis. For example, the Na\(^+\) gradient-induced amino acid movement in cells poisoned with metabolic inhibitors (to reduce the cellular ATP level) was of sufficient magnitude to account for only one third to one half the transport observed in unpoisoned cells possessing Na\(^+\) gradients of similar magnitude (106, 107). Furthermore, small but significant net uphill accumulation of amino acids has been observed in unpoisoned cells having no Na\(^+\) or K\(^+\) gradients (106) and having reversed Na\(^+\) and Na\(^+\) plus K\(^+\) gradients (164, 176, 193). This evidence, though insufficient to render the hypothesis invalid, suggests that a source of energy other than the transmembrane gradients of Na\(^+\) and K\(^+\) is also capable of driving the transport of amino acids.

The present approach to this problem may be described as an experimental examination of several key predictions inherent in the Na\(^+\) gradient hypothesis, particularly those which, if not verified, would expose the contributions of an energy source other than monovalent cation gradients capable of driving net amino acid transport. The main objective was to assess whether or not the Na\(^+\) gradient hypothesis was an adequate description of the net uphill transport of neutral amino acids in Ehrlich ascites cells. Thus experiments were performed which compared the net uphill transport of several neutral amino acids in cells,
(a), containing high and low levels of ATP with monovalent cation
gradients of similar magnitude and (b), where the ATP levels were
fairly constant but the ion gradients varied over a wide range.

Raising the cellular Na\(^+\) concentration to virtually abolish
the normal inward Na\(^+\) gradient had no detectable effect on the
net uptake of methionine and glycine into cells containing ATP
(Tables 5-1 to 5-4). The implication in this finding is clear; if Na\(^+\)
has no detectable effect at the inner surface of the
membrane, then a gradient of Na\(^+\) cannot possibly have any but
a very small effect. However, one may argue that in the pres-
ence of cellular ATP the cellular Na\(^+\) was not homogeneously
distributed within the cell and though the total concentration
of cellular Na\(^+\) was abnormally high, a low concentration of Na\(^+\)
was still present in the immediate vicinity of the plasma mem-
brane. Thus ATP would still have exerted its effects through
a normal Na\(^+\) gradient. This argument can be refuted using the
data in Table 5-11 which shows that in cells possessing similar
"normal" cation gradients, net uphill transport in the virtual
absence of cellular ATP was much less than that in its presence.

Returning to the point, it may be stated that the ineffectiveness
of cellular Na\(^+\) in these cells stands in contrast to its stimu-
latory action on amino acid efflux from pigeon red cells (81,100)
and rabbit small intestine (107).

Lowering the concentration of extracellular Na\(^+\) depressed
the amino acid transport activity when the latter was measured
either in the presence (Tables 5-1, 5-4, 5-12, Figures 5-6 to 5-8) or the absence (Tables 5-9, 5-10, 5-12, Figures 5-5B to 5-8) of cellular ATP. In conjunction with the ineffectiveness of cellular Na\(^+\), this evidence localizes the major effects of Na\(^+\) to the outer surface of the membrane where it stimulates the influx process (Figures 5-5B, 5-7, 5-8). This particular effect of Na\(^+\) on the influx has been repeatedly demonstrated by others (see section 1.2.D. of Chapter 1). Furthermore, the stimulation of influx by Na\(^+\) occurred in the presence or absence of ATP though amino acid uptake was greater in the presence of ATP. Thus one may come to regard this Na\(^+\) stimulation not only as a mechanism which would ensure some transport under low energy conditions, but also as a device which augments the effects of ATP on amino acid transport.

It is apparent that under comparable ion and ion gradient conditions, net amino acid uptake was reduced as the cellular ATP was diminished (Tables 5-2, 5-3, 5-11, Figures 5-6 to 5-8). As previously indicated, it is unlikely that ATP exerted its effects through the Na\(^+\) gradient. One must therefore conclude that ATP or some similar substance is indeed an energy source directly involved in net amino acid transport. In conjunction with the observation that amino acid efflux from these cells is unaffected by ATP (54, 119), the results in Figures 5-7 and 5-8 (which illustrate the effects of ATP and extracellular Na\(^+\) on amino acid influx) localize the effect of ATP to a stimulation
of amino acid influx. Then neither extracellular Na\(^+\) nor ATP affect efflux from these cells but act to increase influx.

That net amino acid uptake under comparable Na\(^+\) gradient conditions was greater in the presence than in the absence of available metabolic energy has also been demonstrated by Eddy (106) and stated by Hajjar et al (107). Eddy attributed the stimulation of uptake to an additional contribution from the K\(^+\) gradient when metabolic energy was available. Jacquez and Schafer (193) examined the correlation between the steady state uptake of α-AIB into Ehrlich ascites cells in the presence of available metabolic energy and the electrochemical potential energy available from various transmembrane Na\(^+\) plus K\(^+\) distributions. Although these authors concluded that Eddy's suggestion was indeed plausible, scrutiny of their observations revealed that when the K\(^+\) gradient was virtually abolished, the total electrochemical potential energy available from the Na\(^+\) plus K\(^+\) gradients fell short of that required to account for the α-AIB transport. Eddy's suggestion therefore appears inconsistent with the experimental evidence which again implies that an energy source other than cation gradients is involved in net amino acid transport.

Hajjar et al (107) maintained that in the absence of available metabolic energy, the normal cation gradients in small intestine quickly dissipated, hence the lowered net amino acid uptake. However, in the present work, net uphill amino acid
transport into Ehrlich cells was estimated over short periods of time during which the dissipation of the cation gradients in ATP-depleted cells was nominal (Table 5-11). Despite the presence of similar normal cation gradients, the amino acid transport was greater in the presence of cellular ATP. The present data are consistent with the conclusion that ATP is directly involved in net amino acid transport. It is of interest to note that ATP apparently had no significant effect on net glycine transport in resealed pigeon red cell ghosts (102).

From consideration of the effects of cellular and extracellular Na\(^+\) and ATP one cannot but conclude that the major portion of the net transport of neutral amino acids in Ehrlich cells is not proportional to the transmembrane Na\(^+\) gradient but is regulated by the level of cellular ATP and/or extracellular Na\(^+\). However, the results in Table 5-10 indicate that Na\(^+\) plus K\(^+\) gradients could cause significant net amino acid flux in a manner predicted by the Na\(^+\) gradient hypothesis when the cells were virtually depleted of ATP. Since this gradient-induced flux in ATP-depleted cells was small relative to transport in ATP-containing cells, one may conclude that, (a), cellular Na\(^+\) does indeed have some limited effect on amino acid efflux which was not detectable in cells containing ATP and (b), cellular K\(^+\) may act to decrease amino acid efflux. The implication of cellular K\(^+\) was prompted by the observation that the combined effects of the Na\(^+\) plus K\(^+\) gradients (Table 5-10),
rather than those of separate Na\(^+\) or K\(^+\) gradients (Table 5-9), was most efficient in generating net uphill amino acid transport in ATP-depleted cells. Thus the Na\(^+\) gradient hypothesis is applicable to only a small portion of net amino acid transport in Ehrlich ascites cells.

The results in Figure 5-6 also indicate only minor involvement of cation gradients in net amino acid transport. The figure shows net amino acid movements in ATP-depleted and ATP-containing cells with fully and partially reversed Na\(^+\) plus K\(^+\) gradients. Net amino acid efflux did not occur unless the cation gradients were fully reversed. Partial (50-60\%) reversal could not stop net amino acid uptake when cellular ATP was present and did not cause any net amino acid movement when cellular ATP was virtually absent. It is apparent that the net transport system(s) for neutral amino acids in Ehrlich ascites cells is arranged so as to use cellular ATP and/or extracellular Na\(^+\) and probably cellular K\(^+\) to produce inward transport under conditions which approach the normal physiological state.

In accepting this point of view, the role of ATP and monovalent cations in the net transport of amino acids becomes apparent. The presence of cellular ATP causes an asymmetry in the amino acid carrier such that influx is increased. Thus, net downhill and uphill transport into the cell are also increased. The presence of extracellular Na\(^+\) also causes an asymmetry in the amino acid carrier such that influx is increased.
As this Na\(^+\)-induced asymmetry is unaffected by the presence or absence of ATP, it can either augment the stimulation of influx due to ATP or it can itself stimulate influx in the virtual absence of ATP. The presence of cellular K\(^+\), in all probability, decreases amino acid efflux thus further augmenting the effects of ATP and/or extracellular Na\(^+\). One may liken this transport system to a mechanism such as Model 2 (see section 1.2.A. of Chapter 1) when, (a), ATP is required for the Z to C but not the C to Z conversion, (b), extracellular Na\(^+\) acts as a stimulating co-substrate in amino acid influx, (c), cellular K\(^+\) probably acts as an inhibitor of amino acid efflux, (d), the influx of amino acid can be catalyzed by Z,C,ZNa\(^+\) and CNa\(^+\) but the influx catalyzed by Z < ZNa\(^+\) < C < CNa\(^+\) and (e), the association between Na\(^+\) and carrier is the same when the carrier is in the C or Z form.

In summary, the concept of the Na\(^+\)-sensitive, neutral amino acid net transport system(s) which emerged from the present evidence is more complex than that of the simple facilitated diffusion system which stands at the root of the Na\(^+\) gradient hypothesis. It encompasses a membrane-bound, enzyme-like system the stability of which is unaffected by transient depletion of cellular ATP and cessation of protein synthesis. It is capable of using ATP, extracellular Na\(^+\) and (probably) cellular K\(^+\) to stimulate net amino acid transport into the cell. Furthermore, it is a system which is strongly oriented towards inward net
uphill transport. The reason for this strong orientation may be more readily understood if one considers that these cells not only grow and divide rather quickly (and therefore maintain a sizable endogenous free amino acid pool to satisfy the demands of continuous rapid protein synthesis) but they also possess a comparatively leaky plasma membrane. For example, poisoning tumor cells, erythrocytes, muscle tissue or squid axon with metabolic inhibitors to deplete cellular ATP results in the loss of the physiological membrane potential, Na⁺ and K⁺ distributions. However, the loss of these from Ehrlich ascites cells occurs at a rate measureable in minutes while the loss from the other tissues is measured over a period of hours and days. Indeed, if the plasma membrane of Ehrlich cells were not so leaky, it would have been impossible to manipulate the cellular ionic (Tables 3-1, 5-6 to 5-8) and amino acid (Table 4-1) composition with such facility and within such short periods of time.

6.2 EXCHANGE DIFFUSION AND NET TRANSPORT

Exchange diffusion and net transport are two different means of transmembrane transfer common to the mechanism of carrier mediated transport and can be catalyzed by the same carrier. Unlike the process of net transport, that of exchange diffusion does not result in a net change in the total concentration of substrate on either side of the membrane thus eliminating the
thermodynamic requirement for driving forces such as cation
gradients, ATP, etc. However, if combination with cations or
ATP is required for the carrier to perform its function at a
measurable rate, then net transport, counter-transport and ex-
change diffusion should all exhibit those requirements. For
example, in pigeon red blood cells and rabbit reticulocytes,
both net transport and exchange diffusion of alanine are medi-
ated by the same system which requires Na\(^+\) (91,199).

The results in Chapter 3 clearly indicate that the process
of methionine exchange diffusion in Ehrlich ascites cells is
unaffected by Na\(^+\), K\(^+\), their transmembrane gradients or ATP.
In view of the action of Na\(^+\), K\(^+\) and ATP on the net transport
of neutral amino acids in these cells, these results suggest
either that the exchange diffusion and net transport of methi-
onine are catalyzed by the same system but show different ionic
and energy requirements or that the two processes are mediated
by distinct systems each with its own particular requirements.
The latter suggestion is more consistent with evidence previ-
sously reported by Christensen and co-workers. For example,
studies concerned with the exchange diffusion of neutral amino
acids and their inhibitory interactions during uptake into
Ehrlich ascites cells revealed that, (a), neutral amino acids
could be clustered mainly into two overlapping groups on the
basis of their inhibitory interactions (48), (b), the members
of one of these groups participate mainly in exchange and
heteroexchange diffusion (47,48), and (c), the uptake of the members of the other group showed a very much more marked sensitivity to extracellular pH and Na\(^+\) (48,146,161). Thus it was concluded that the system which primarily mediates exchange diffusion of neutral amino acids in Ehrlich cells (the L system) is distinct from that (or those) which operate predominantly for net transport (48,161).

Exchange diffusion of neutral amino acids in these cells is a process mediated by a mechanism which will function at full efficiency not only under physiological conditions, but under many adverse conditions excluding those which result in damage to its structural integrity. As noted above, the net transport systems cannot function at full efficiency when ATP and/or extracellular Na\(^+\) are limiting. However, under physiological conditions, one would expect both net transport and exchange diffusion systems to be operative. If one were to measure the uptake of neutral amino acids which were substrates for both types of systems, it would be difficult to assess what portion of the total uptake would be a result of net transport and what portion would be due to exchange. Difficulties in distinguishing between net transport and exchange diffusion originally led to the conclusion that the predominantly exchanging L system in Ehrlich ascites cells was capable of catalyzing net uphill transport (48). The conclusion was based on the observation that neutral amino acids handled predominantly by the L
system were apparently accumulated in the cells to a concentration greater than that in the extracellular medium.

In the present work, the contribution of the exchange system to the total methionine uptake was estimated directly; experiments were performed which, (a), assessed the changes in the endogenous free amino acid pool during methionine uptake and (b), assessed the effects of several other neutral amino acids and (c), the depletion of cellular ATP and endogenous free amino acids on the total uptake of methionine. Methionine was used as the substrate because it participates in both net transport and exchange diffusion in Ehrlich cells.

Methionine uptake was accompanied by the loss of several amino acids from the endogenous free amino acid pool (Table 4-2). These amino acids are known to participate in heteroexchange diffusion with methionine. The loss was approximately equal in magnitude to the total methionine uptake from Na⁺-free media (at 25°C) at 2 minutes of incubation. Although no further methionine-induced loss was detected after 2 minutes of incubation, the total methionine uptake increased and at 60 minutes, was 2.5 times the level it had been at 2 minutes. Furthermore, amino acids which do not exchange with methionine (such as α-AIB and alanine) did not inhibit the total methionine uptake during the first 2 minutes of incubation but caused a substantial inhibition at later times. These results clearly indicate, (a), that two processes are involved in the total methionine uptake from
Na\(^+\)-free media, (b), that at early times, the total uptake is due largely to an exchange between extracellular methionine and several components of the endogenous free amino acid pool, and (c), that the later uptake is probably due to net transport which is mediated by a carrier distinct from that catalyzing exchange diffusion. Furthermore, the appearance of two kinetic components in the total initial uptake (Figure 4-3) is consistent with the conclusion that both net transport and exchange diffusion are simultaneously operative.

The detection of two components in the total methionine uptake at early times from Na\(^+\)-free media was also reported by Inui and Christensen (161) who made no further attempt to identify those components. These authors also referred to the methionine uptake from Na\(^+\)-free media as "concentrative", implying net uphill transport to have occurred via the L system. The present evidence confirms that net uphill transport of methionine does occur in the absence of extracellular Na\(^+\), but also indicates that the net uphill transport was not due to the activity of the exchange carrier (L system). This conclusion was formulated using the following evidence: (a), in the absence of extracellular Na\(^+\), net methionine uptake in excess of the uptake due to exchange diffusion was inhibitable by \(\alpha\)-AIB (Figure 5-2A), (b), it was this \(\alpha\)-AIB inhibitable component which raised the cellular methionine concentration to a level exceeding that in the extracellular fluid, and (c), \(\alpha\)-AIB does not interfere with the activities of the exchange carrier (48).
Therefore it was not the exchange carrier (L system) which was responsible for the observed net uphill transport. Furthermore, as α-AIB does show relatively strong affinity for the net transport system(s) (48) and inhibits net methionine uptake from Na⁺-free medium, one may conclude that the net transport systems retain some activity in the absence of extracellular Na⁺. This last statement is at variance with the views of Inui and Christensen (161) who have stated that the Na⁺-insensitive methionine uptake is not produced by residual operation of the Na⁺-requiring system(s) handicapped by the absence of Na⁺. However, their views were based on measurements of the total methionine uptake at early times which failed to distinguish between uptake due to exchange diffusion with endogenous amino acids and uptake due to net transport.

Further evidence that the exchange carrier (L system) does not mediate net transport comes from Table 4-4 which shows the methionine uptake from Na⁺-free media into cells virtually depleted of ATP and endogenous amino acids. As (a), lack of Na⁺ and ATP seriously hampered the net transport systems but did not interfere with the activity of the exchange carrier and (b), the possibility of uptake due to exchange diffusion was eliminated by the removal of the endogenous amino acids, net transport due to the activity of the exchange carrier should have been apparent. However, methionine uptake was barely detectable and equilibration of methionine between medium and cell
was not achieved in 2 hours. It was therefore concluded that the carrier catalyzing exchange diffusion of neutral amino acids in Ehrlich ascites cells does not mediate net transport. Indeed, one may liken this system to a mechanism such as Model 1 (section 1.2.A. of Chapter 1) when the translocation of the free carrier through the membrane is the rate-limiting step and is very slow. Thus, the system could catalyze exchange diffusion but hardly any net transport.

One may assign a rather important role to the L system in these cells if one considers that, (a), the substrates normally handled by this system do not show great affinity for the net uphill transport systems and are consequently not well accumulated, (b), these substrates include 8 of the 10 essential amino acids, and (c), this mechanism provides a means of rapid exchange of amino acids present in the cell at excessive concentrations for other amino acids present in the extracellular fluid. Thus the L system would appear to exert some control over the composition of the endogenous free amino acid pool and so may aid in filling the demands for particular substrates without which growth could not occur.
CLAIMS TO ORIGINAL RESEARCH

1.a. The effect of temperature on the process of methionine exchange diffusion was examined in the range between 0° and 15°C. The thermal activation energy (Arrhenius E value) for that process was found to be approximately 40,000 calories/mole.

b. The exchange diffusion of methionine was found to be insensitive to, (a), changes in the levels of cellular Na⁺ and K⁺, (b), changes in the transmembrane gradients of those cations and (c), prior depletion of cellular ATP.

2. The composition of the endogenous free amino acid pool of saline-washed Ehrlich ascites cells was determined after the cells were incubated in, (a), normal Krebs-Ringer medium, (b), a modified Ringer solution wherein K⁺ replaced the Na⁺ and containing 10⁻⁴ M DNP and (c), normal Krebs-Ringer medium followed by a second incubation -- in the presence and absence of 2 mM L-methionine -- in a modified Ringer solution wherein choline⁺ replaced the Na⁺. The incubation in (b) above drastically decreased the endogenous pool while incubation as in (c) above, but in the presence of extracellular methionine, resulted in decreased levels of only those amino acids known to participate in exchange diffusion with methionine.
3. The uptake of L-methionine into Ehrlich ascites cells in the absence of extracellular Na\(^+\) was examined. This uptake consists of two components, one of which is inhibitable by α-AIB and L-alanine. The inhibition of methionine uptake caused by these two amino acids becomes apparent only after the first few minutes of incubation. The portion of the methionine uptake sensitive to these two amino acids appears to be a net transport process. However, the α-AIB, L-alanine-insensitive component is not net uptake but exchange diffusion between extracellular methionine and several members of the endogenous free amino acid pool.

4. When Ehrlich ascites cells are depleted of ATP and endogenous free amino acids and subsequently placed in a Na\(^+\)-free medium containing methionine, they do not equilibrate the amino acid between the extracellular and cellular compartments within a 2 hour period. This result indicates that the carrier catalyzing exchange diffusion of neutral amino acids (L system) -- which is unaffected by ATP depletion and lack of Na\(^+\) -- is virtually incapable of mediating net transport.

5.a. The magnitude of the transmembrane Na\(^+\) gradient was found to have little or no effect on the net uptake of glycine and methionine provided that the cells contained near
normal concentrations of ATP.

b. In cells depleted of ATP, the transmembrane $\text{Na}^+$ gradient had some effect on the influx or efflux of methionine. Gradients of $\text{Na}^+$ and $\text{K}^+$ separately induced only nominal net uphill transport of methionine into the cell. However, the $\text{Na}^+$ plus $\text{K}^+$ gradients, when combined, were more efficient in producing net uphill transport of glycine, methionine and leucine both into and out of the cell. This gradient-induced flux was of small magnitude and did not approach the rate or degree of amino acid transport observed under more physiological conditions.

6.a. The presence of cellular ATP greatly stimulated the net uptake of glycine, methionine and leucine in cells with similar $\text{Na}^+$ plus $\text{K}^+$ gradients. ATP exerts its stimulatory effect on glycine and methionine transport by decreasing the apparent Michaelis constant for the influx process while leaving the apparent maximum influx unchanged. Furthermore, the presence or absence of cellular ATP did not change the stimulatory effect of extracellular $\text{Na}^+$ on the influx of these amino acids.

b. The $\text{Na}^+$ gradient hypothesis was found to be applicable only to a very small portion of the net transport of neutral amino acids in Ehrlich ascites cells.
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