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KINETICS OF AMINO ACID TRANSPORT ACROSS BONE MARROW CELL MEMBRANES

Max Shiuming Lin

(Ph. D. Thesis)

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KINETICS OF AMINO ACID TRANSPORT ACROSS BONE MARROW CELL MEMBRANES

Max Shiuming Lin

ABSTRACT

Radioisotopic tracer is a powerful tool for studying cellular metabolism. Following administration of a labeled precursor to intact cells or animals, appearance of label in products can be measured. Clearly, quantitative interpretation of such measurements requires quantitative information concerning transfer of the precursor across cell membranes. Cell membrane transport can be studied relatively readily in isolated cell systems under controlled conditions. Information gained from such *in vitro* studies may provide valuable insight into *in vivo* processes.

I have studied the kinetics of amino acid transport and intracellular amino acid pools in dog bone marrow cells *in vitro*. It is anticipated that techniques developed for these cells in the work can be adapted for related studies with human blood and bone marrow cells.

Dog bone marrow nucleated cells were incubated in media containing labeled L-amino acids, and the cellular accumulation of radioactivity as a function of time was measured and analyzed according to a three-compartmental model. In the

compartmental analysis, an optimization program was used on a digital computer to fit the data of radioactivity uptake with a function derived from the model. From the least squares best fit results, it was possible to evaluate both cell membrane transport and intracellular pool turnover of the amino acids studied.

The turnover halftime of intracellular histidine arising from extracellular sources was 6.0 ± 0.7 (SEM) min. Similar results were obtained for serine, tryptophan, and methionine. Loss of the amino acids to extracellular pools accounted for a major portion of this turnover. These results indicate that subsequent to the administration of labeled amino acids the rate of equilibration between the extracellular and intracellular pools constitutes an important "rate-limiting step" in the production of labeled metabolites. α-N-formiminoglutamate added to the culture was inferior to histidine as a source of cellular monocarbon fragments, because the former accumulated inside the cell much more slowly than the latter.

Histidine, serine, tryptophan, and methionine were all found to be actively transported into the cell. Histidine appeared to enter the cell by both a facilitated process with an apparent Michaelis constant of 0.28 mM and a limiting flux of 14×10^{-8} mμmole/min per cell, and an apparent first order process not solely accountable by simple diffusion. At physiologic extracellular histidine concentrations,

histidine entered the cell predominantly by the former process. Loss of histidine from the cell appeared to be substantially facilitated, but exhibited an apparent first order process at 0-13 mM intracellular histidine concentrations, and thus appeared to have an apparent Michaelis constant much greater than that of histidine entry.

Insulin and glucagon had no measurable effect on histidine transport. Methionine depressed the rate of entry into the cell, the fractional rate of loss from the cell, and the fractional turnover rate of intracellular pool of both histidine and serine.

Pyridoxal administered at 2.5 mM concentration to the culture medium seemed to cause acceleration of rates of both entry into and exit from the cell and hypertrophy of intracellular pools of both histidine and tryptophan. It was found incidentally that ammonium oxalate had diminished hemolytic effect on erythrocytes previously exposed to the 2.5 mM pyridoxal. These erythrocytes appeared to have a slightly decreased osmotic fragility apparently independent of possible small changes in the cell volume prior to fragility test in hypotonic saline solutions.

I. INTRODUCTION

Incorporation of label into a metabolite from a labeled amino acid administered to intact cells depends upon its transport across the cell membrane as well as upon its metabolism within the cell. Measurement of appearance of label in metabolites may reflect primarily the rate of entry of the amino acid into the cell and the rate of equilibration between its extracellular and intracellular pools rather than the rate of its actual metabolism. Similarly, the effect of a physiologic or pharmacologic agent on such measurement may arise primarily at the level of cell membrane transport of the amino acid. Under these circumstances, quantitative interpretation of data describing the incorporation of label from a labeled amino acid into other metabolites requires knowledge of the kinetics of transport and equilibration between its extracellular and intracellular pools.

Little information is available on the kinetics of intracellular amino acid pools. Published studies in various mammalian tissue slices, intestines, and Ehrlich ascites tumor cells primarily concern transport mechanisms in these systems (1,2). Amino acid transport apparently has not been studied in bone marrow cells. This paper presents kinetic studies of amino acid transport and intracellular amino acid pools in the nucleated cells of dog bone marrow.

II. KINETICS OF AMINO ACID TRANSPORT AND INTRACELLULAR AMINO ACID POOLS IN DOG BONE MARROW CELLS

A. INTRODUCTION

1. Some Terms and Concepts in Transport across Cell Membranes

In this paper, transport signifies the transfer of solutes across the cell membrane. Flux designates the rate of a one-way movement. Influx and efflux refer to the rate of entry into and loss from the cell, respectively. The flux of a solute transported by simple diffusion is proportional to its electrochemical potential on that side of the membrane from which the flux is directed. For a solute bearing no net charge, this flux is approximately proportional to the concentration of the solute (Fick's Law).

Frequently, saturation phenomenon is observed in transport. As the electrochemical potential or concentration of a solute increases, its flux eventually reaches a maximum value. This suggests that a chemical reaction with certain structural site or sites mediates the transport of the solute. This transport operates on existing electrochemical gradient of the solute, leads to disappearance of the gradient, and hence is called mediated or facilitated diffusion (1). When the electrical potential gradient can be ignored, the flux by facilitated diffusion is describable empirically by the Michaelis-Menten equation of an enzyme kinetics:

$$V = V_{max}S/(S + K_m) \qquad (1)$$

where S is the concentration of the solute on the side from

which the flux V is directed; K_m, the apparent Michaelis constant; and V_{max}, the limiting flux.

Stein (1) has listed defining characteristics of facilitated diffusion. These characteristics include, in addition to those already mentioned, the following: (a) If the solute is optically active, its optical enantiomorph is likely to have a very different rate of transport. By simple diffusion, these rates should not differ. (b) Inhibition of the transport of a solute by the presence of its structural analogs, i.e., the phenomenon of <u>competition</u>. (c) Apparent capability of transport in one direction against existing electrochemical gradient of the solute when its transport in the opposite direction down the gradient is inhibited by structural analogs, i.e., the phenomenon of <u>counter-transport</u> or <u>counterflow</u>. The violation of Fick's Law of simple diffusion, the exhibition of analog competition, and the manifestation of counterflow are all considered strong evidences for a facilitated diffusion. Such consideration is valid if it can be shown that in the range of concentrations of the solute or its analogs used the cell membrane is not modified to any extent. When the transport of appropriate control solutes is not likewise affected, the membrane may be considered unmodified.

<u>Active transport</u> is a term reserved for transport uphill against electrochemical gradient of the solute. A sufficient criterion is that the flux ratio departs from the

concentration ratio between the two sides of the membrane---when the ratio influx/efflux is greater than the concentration ratio extracellular/intracellular, the solute is said to be actively transported into the cell; when the former ratio is smaller than the latter ratio, it is said to be actively transported out of the cell. Since influx and efflux of a solute are often nearly equal to each other at equilibrium, a frequent consequence of active transport directed into the cell is that the equilibrium intracellular concentration of the solute is higher than its extracellular concentration, i.e., the distribution ratio is greater than unity.

2. Amino Acid Transport

The entry of neutral L-amino acids into certain mammalian cells obeys the criteria of a facilitated diffusion system. The influx as a function of extracellular concentration can be described by equation 1. In addition to this saturable component, an apparently nonsaturable component, first order with respect to concentration, has been observed for several neutral L-amino acids in erythrocytes (3,4) and for α-aminoisobutyric acid (AIB) in Ehrlich ascites tumor cells (5). The first order constant is usually referred to as apparent diffusion constant (k_d) since the first order component may represent a simple diffusion process or at least one facilitated system possessing a high K_m or both.

In recent years, the concept of co-transport of an amino acid together with sodium ions has emerged to explain those

cases of amino acid transport which depend on simultaneous transport of sodium ions. Working with pigeon red cells, Vidaver (4) found that the saturable component of glycine influx was strictly dependent upon the concentration of sodium ion in the culture medium. At a given glycine concentration, slower glycine influx was associated with lower sodium ion concentration. Lithium ion could not replace sodium ion in driving the glycine influx and absence of potassium ion from the medium was not deleterious. The other (first order) component of glycine influx was unaffected by the variation in sodium ion concentration. Interestingly, the K_m of the saturable component appeared to be dependent upon the square of sodium ion concentration:

$$K_m = C_1/(Na^2 + C_2) \qquad (2)$$

where C_1 and C_2 were some constants. V_{max} was unaffected. Direct measurement of both glycine influx and sodium ion influx appeared to substantiate an anticipated stoichiometric ratio of 2 moles of sodium ion for each mole of glycine transported into the cell. These quantitative results were later found to be not generally applicable, but qualitatively similar results were obtained for alanine and AIB in pigeon red cells and in Ehrlich ascites tumor cells by Wheeler, Inui, Hollenberg, Eavenson, and Christensen (6). Additional support for the co-transport concept comes from the work of Eddy and Mulcahy (7). They showed that even in the presence of 2 mM sodium cyanide, when adenosine

triphosphate was no longer available, Ehrlich ascites tumor cells were still able to concentratively accumulate glycine provided there was an inwardly directed sodium ion gradient. The foregoing and other related findings have led to the following postulate (2):

Active transport of amino acids into the cell may result from a co-transport process which requires attachment of both the amino acid and the sodium ion to the same transport site or carrier. A complex formed by the amino acid, the sodium ion, and the carrier appears to be the diffusing unit. Sodium ion is actively extruded from the cell by a primary active transport creating an electrochemical gradient of sodium ion, which then drives the secondary active transport of amino acids into the cell by co-transport of the amino acid together with the sodium ion.

In a given cell type, an amino acid may enter the cell by more than one facilitating system. An extensive work in Ehrlich ascites tumor cells by Oxender and Christensen (8) best illustrates this point. By observing the extent to which each of a number of neutral L-amino acids inhibits the uptake of others, they have demonstrated the existence of at least two different facilitating systems with overlapping specificities for neutral amino acids. One system, the A system, had good affinities for alanine, AIB, glycine, serine, threonine, proline, glutamine, and methionine while the other system, the L system, prefers leucine, isoleucine, valine,

phenylalanine, and, again, methionine. The overlap in specificities between the two systems was so extensive that all neutral amino acids, except perhaps glycine, were transported by both systems. The A system appeared to be strongly dependent on sodium ion concentration of the incubation medium while the L system, weakly so.

It may be readily appreciated that the whole story of amino acid transport is a complex one. Are those facilitating systems characterized in terms of entry one-way or two-way streets? How is the exodus of amino acid from the cell accomplished? How fast are the intracellular free amino acids turning over? These and many other problems demand further investigations.

3. Measurement of K_m, V_{max}, and k_d

As previously mentioned, an amino acid transport can frequently be characterized in terms of K_m, V_{max}, and k_d as if the amino acid were transported by both a facilitated process and a first order process. This is stated by the following equation describing the amino and influx (I) as a function of its extracellular concentration (5):

$$I = k_d S + V_{max} S (S + K_m)^{-1} \qquad (3)$$

From a functional point of view, the characterization in terms of these kinetic constants serves to identify the transport systems involved and also to indicate their relative importance in a given amino acid transport.

Knowledge of these kinetic constants is important. Suppose the influx of an amino acid is unaltered by an agent

in a controlled experiment. From such experiment, it is impossible to conclude that the agent has no effect on the amino acid entry unless the control values of K_m, V_{max}, and k_d are known. The agent might have altered the affinity of the facilitating system for the amino acid and hence altered the K_m. If the extracellular concentration of the amino acid used in the experiment happens to be much higher than the K_m, the facilitated component of the influx may not be substantially altered by an alteration in K_m as may be seen from equation 1. Furthermore, even a substantial change in the facilitated component may not give a detectable change in the influx unless the component accounts for a substantial fraction of the influx.

Experimental measurement of these kinetic constants for entry is a simple matter conceptually. One would only have to secure data of influx at various concentrations of the amino acid in the incubation medium and analyze the data according to equation 3. Winter and Christensen (3) have employed a graphical method for the analysis. The data of influx were plotted against the concentration. k_d was identified with the slope of the terminal straight portion of the plot. Then k_dS term was subtracted from I and the reciprocal of the difference was plotted against the reciprocal of S to determine V_{max} and K_m (Lineweaver-Burk plot). An objection to this graphical method is the conjectual element involved in finding the terminal slope of the plot of I versus S.

In practice, measurement of the amino acid influx poses technical problems. What can be measured, chemically or radioisotopically, is an uptake (net entry) of the amino acid or label during a finite incubation time. A short incubation time, often a matter of one or two minutes, during which exodus of the amino acid or label is negligible compared to their entry is much too short compared to the time required to accomplish isolation and washing of cells subsequent to the incubation.

To estimate kinetic constants for amino acid exodus, it has been necessary, as in the work of Christensen and Handlogten (9), to resort to "reverse" experiments in which cells are first loaded with the amino acid and the efflux is then measured. Relatively little work has been done on amino acid exodus apparently because precision of measurement by available methods is even worse for efflux than for influx.

For the measurement of influx and efflux, radioisotopic method is superior to chemical method for two reasons: (a) Chemical assay requires prior extraction of the acid-soluble fraction from the cell and sometimes chromatographic separation of the amino acids in the extract also. Radioisotopic assay can be done simply on whole cells. (b) Since natural amino acids can be incorporated into proteins, chemical assay tends to underestimate their influx and overestimate their efflux.

4. Purpose of This Study

In the present work, dog bone marrow cells were incubated for varying intervals in the presence of labeled L-amino acids. For each of the amino acids studied, the intracellular accumulation of radioactivity by bone marrow nucleated cells was analyzed according to a three-compartment model. From such analyses, the influx and efflux of the amino acid and the fractional turnover rate of its intracellular pool were calculated. By analysis of the calculated histidine influx as a function of extracellular histidine concentration in accordance with equation 3, the relative role of active and passive transport in histidine entry was evaluated.

Dog bone marrow cells were chosen for this work, because it was felt that a method developed for these cells might find future application to nucleated cells in human bone marrow and peripheral blood. The compartmental analysis presented in this study made possible concurrent evaluation of both entry and exodus processes and intracellular pool turnover from "forward" experiments alone. For this reason, it was believed to be superior to conventional methods for studying the kinetics of amino acid transport.

B. MATERIALS AND METHODS

1. Materials

Labeled L-amino acids, tritiated water (HTO), and inulin-carboxyl-^{14}C were obtained from Nuclear-Chicago Corp. (Des Plaines, Ill.) or New England Nuclear Corp. (Boston, Mass.); L-amino acids, from Calbiochem (Los Angeles, Calif.);

Gey's Balanced Salt Solution (Gey's BSS) and Basal Medium of Eagle with L-glutamine (BME), from Grand Island Biological Co. (Grand Island, N.Y.); heparin without preservatives, from Abbott Lab. (North Chicago, Ill.); dextran (average mol wt 250,000), from Pharmacia (Uppsala, Sweden); ammonium oxalate, from General Chemical Co. (New York, N.Y.); methotrexate, from Lederle Lab. (Pearle River, N.Y.); sodium dodecyl sulfate (SDS), from K and K Laboratories, Inc. (Plainview, N.Y.); and formic acid (88% w/w or higher), from Allied Chemical Corp. (Morristown, N.J.). Heparin was dissolved in the medium; ammonium oxalate, in distilled water; and SDS, in 45% (v/v) ethanol.

2. Whole Cell Uptake of Radioactivity

In each experiment, bone marrow cells were aspirated from the femurs of 2-3 lightly anesthetized dogs (sodium pentobarbital), anticoagulated, pooled, and subjected to dextran-sedimentation to eliminate approximately 99% of the erythrocytes present. After centrifugation of the supernatant fluid in conical tubes at 450 x g for 3-4 min, approximately the upper three quarters (less contaminated by erythrocytes) of the sediment formed were resuspended in appropriate medium containing heparin (60 U/ml). The suspension was passed through a mesh (Macalaster Bicknell Co., Cambridge, Mass., Cat. No. 9331) to remove cell aggregates. Cell counting was done in duplicate with a Coulter Counter (Coulter Electronics, Chicago, Ill.). In the final suspension, there was one nucleated cell for every 1-3 erythrocytes

present. By morphological characteristics, about 20-30% of the nucleated cells were deemed capable of further mitosis. Two ml portions of the cell suspension were delivered with volumetric pipets into a series of incubation tubes (Nalge Company, Rochester, N.Y., Cat. No. 21009-400) containing known quantities of amino acids. Following equilibration of the suspension at 37°C for 20-30 min, tracer quantities of labeled amino acids were added in equal amounts to all tubes without appreciably changing the concentration of amino acids. The volume of incubation was 5 ml in most experiments. The incubation was carried out at 37°C with shaking under air for 0-2 hours. The number of nucleated cells remained essentially constant during the incubation as previously shown for human marrow cells under similar conditions (10). Gross hemolysis was absent. The extracellular concentration and specific activity of amino acids were kept essentially constant by using low cell concentration (1% v/v or less) and short incubation time (11,12). In most experiments, the maximum cellular uptake of radioactivity was less than 3% of that given to the culture. After addition of 5 ml iced medium to the culture, the nucleated cells were harvested by centrifugation in a Sorvall RC2-B centrifuge (Ivan Sorvall Inc., Norwalk, Conn.) at -3°C and 2,500 x g for 4 min, decantation of the supernatant fluid, resuspension of the packed cells in 8 ml iced 80 mM ammonium oxalate, recentrifugation of the suspension in a conical glass tube at 1,200 x g for 3 min, and decantation of the resultant supernatant hemolysate.

During the single washing with ammonium oxalate solution, most of the contaminating erythrocytes were lysed. The entire procedure from the dilution in iced medium to the recovery of nucleated cells took 10 min. Termination of incubation was taken to be at 1 min after the dilution. The entire cell pellet was digested in 3 ml NCS Solubilizer (13), and 10% aliquot of the digest was assayed for radioactivity with a Nuclear Chicago Mark I Liquid Scintillation Computer. Where double labeling was employed, simultaneous equations were solved for the individual isotope dpm after determining their counting efficiencies at the two characteristic channels by internal standardization with ^{3}H- and ^{14}C-toluene. Counting efficiencies were uniform among the samples in any given experiment.

3. Fractionation of Whole Cell Radioactivity

Some pellets of harvested cells were subjected to extraction with iced trichloroacetic acid (TCA, 5% w/v) for 30 min and then with hot (88°C) TCA for 20 min. The radioactivity in these TCA extracts and TCA precipitate was determined by liquid scintillation counting.

4. Specific Activity of DNA Thymine

Two ml portions of the cell suspension were delivered into four incubation tubes containing histidine-imidazole-2-^{14}C or -N-formiminoglutamic acid-formimino-^{14}C (FIGLU-$^{14}C_1$) with or without methotrexate. After 1 or 2 hour incubation in the metabolic shaker at 37°C, the sodium salt of DNA (Na-DNA) was isolated by a modification of the method of

Kay, Simmons, and Dounce (14). Following the addition of iced medium to the culture, cells were packed by centrifugation at -3°C, washed twice in iced 80 mM ammonium oxalate and then once in 0.14 M NaCl, and blendered in 0.14 M NaCl using a Sorvall Omni-Mixer Microhomogenizer immersed in ice. The homogenate was centrifuged at -3°C and 27,000 x g for 30 min. The supernatant fluid containing dissolved ribonucleoproteins was discarded. The sediment was resuspended in 2.7 ml 0.14 M NaCl, and 0.3 ml 5% (w/v) SDS was added to the suspension. The mixture was stirred for 1 hour to dissociate the nucleoprotein complex into the nucleic acid and the protein and also to precipitate the protein. Then the mixture was brought to 1 M with respect to NaCl concentration and centrifuged at -3°C and 27,000 x g for 30 min. The grayish fibrous mass formed during gradual addition of equal volume of 95% (v/v) ethanol to the separated supernatant fluid was wound up around the end of a stirring rod. This crude Na-DNA was immediately dissolved in 2.7 ml distilled water. Then followed the same addition of SDS, stirring, adjustment of NaCl concentration, centrifugation, and recovery of fibrous mass, as the corresponding steps previously mentioned. The mass was washed in 95% (v/v) ethanol and then in acetone once each and dried. This Na-DNA preparation was then hydrolyzed in a sealed pyrex glass tube with 0.5 ml formic acid at 175°C for 35 min (15,16). The entire hydrolysate was concentrated and then spotted on Whatman #1 paper for separation of the bases present by descending chromatography in an isopropanol-

HCl solvent system (17). Thymine was eluted with 0.1 N HCl from the paper after locating its spot under an UV lamp (16). Specific activity of the thymine was calculated from thymine concentration and radioactivity concentration of the eluate. The former was determined by UV spectrophotometry at the absorption maximum (266 mu) with a Beckman DU Spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) and the latter, by liquid scintillation counting.

5. Intracellular Water Space

Cell suspensions with nucleated cell/erythrocyte ratios greater than 2 were prepared by following the dextran-sedimentation procedure with two successive "differential centrifugation" steps. Following 20 min incubation in the presence of both HTO and inulin-carboxyl-^{14}C, a known quantity of cells was packed by centrifugation at 1,200 x g for 10 min. ^{3}H and ^{14}C activities in the cell pellet as well as their concentrations in the supernatant fluid were determined by liquid scintillation counting with precautions taken against HTO loss from samples by evaporation. From these results, the HTO space and the inulin-carboxyl-^{14}C space of the cell pellet were calculated. The intracellular water space (W) of the nucleated cells present in the pellet was taken to be the difference between the two spaces. Such calculations were felt to overestimate water content of the nucleated cells because of the contaminating erythrocytes in the pellet.

6. Statistical Methods

Student's t test was used for testing significance of difference between two means.

C. COMPARTMENTAL ANALYSIS

The cellular uptake of radioactivity is analyzed according to the compartmental model illustrated in Fig. 1. The assumptions inherent in this model are as follows: (i) Labeled amino acids are introduced into the extracellular fluid at zero time without appreciably disturbing the prevailing steady state. The concentration and the specific activity of the extracellular amino acid are kept essentially constant thereafter. (ii) When a labeled amino acid is metabolized in the cell, the label is translocated to other molecular species. For most labeled amino acids, the translocated label appears principally in proteins (18). Further, the estimated order of magnitude for the fractional turnover rate of various mammalian cellular proteins *in vitro* and *in vivo* is 1 %/hour (11). Thus, during a short incubation period, most of the translocated label remains in the cell and the recycling of label back to the intracellular amino acid pool via amino acid biosynthesis and protein catabolism is negligible compared to the steady known inflow of labels from the extracellular pool. Under these circumstances, the function describing the radioactivity uptake versus time has a simple form in terms of the fractional rates as derived below.

For a given amino acid, $A_1(t)$, $A_2(t)$, and $A_3(t)$ denote the amount of radioactivity in compartment I (extracellular pool), II (intracellular pool), and III (translocated), respectively; k_{12}, k_{21}, and k_{23} are the fractional rates as indicated in Fig. 1. $A_1(t) = A_1(0)$ approximately. $A_2(0) = A_3(0) = 0$. The radioactivity influx (R) and the fractional

FIGURE 1

Compartmental model for analysis of intracellular accumulation of labels from extracellular labeled amino acids. It is assumed that intracellular metabolism of the labeled amino acid results in translocation of labels primarily to substances which do not leave the cell at significant rate and that recycling of labels back to the intracellular amino acid pool via biosynthesis and reutilization is negligible compared to the entry of labels from the extracellular pool. k_{12}, k_{21}, and k_{23} represent the fractional rates of entry, exit, and metabolism of the labeled amino acid, respectively; A_1, A_2, and A_3 represent the amounts of the amino acid label in compartments I (extracellular pool), II (intracellular pool), and III (translocated), respectively.

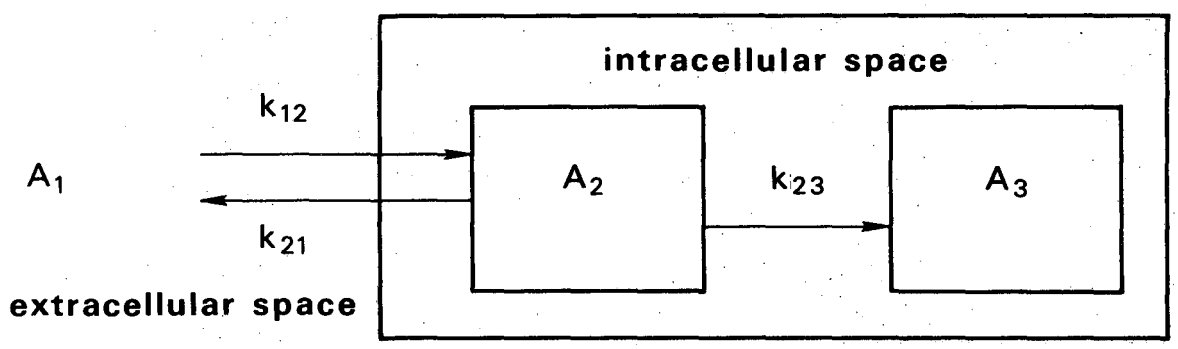
intracellular space
k_{12}
A_1
A_2
k_{23}
A_3
k_{21}
extracellular space

Fig. 1

turnover rate of the intracellular pool (r) are then given by

$$R = k_{12}A_1(0) \tag{4}$$

$$r = k_{21} + k_{23} \tag{5}$$

Then $$dA_2/dt = R - rA_2(t) \tag{6}$$

$$dA_3/dt = k_{23}A_2(t) \tag{7}$$

The two differential rate equations are readily solved to give

$$A_2(t) = (1 - e^{-rt})R/r \tag{8}$$

$$A_3(t) = (k_{23}R/r)t - (1 - e^{-rt})k_{23}R/r^2 \tag{9}$$

The sum of $A_2(t)$ and $A_3(t)$ gives the radioactivity uptake, $A_c(t)$,

$$A_c(t) = A_2(t) + A_3(t) = K_1 + K_2t - K_1e^{-rt} \tag{10}$$

where $$K_1 = (R/r) - (k_{23}R/r^2) \tag{11}$$

$$K_2 = k_{23}R/r \tag{12}$$

Solving equations 4, 5, 11, and 12 for the three fractional rates,

$$k_{12} = (rK_1 + K_2)/A_1(0) \tag{13}$$

$$k_{21} = r^2K_1/(rK_1 + K_2) \tag{14}$$

$$k_{23} = rK_2/(rK_1 + K_2) \tag{15}$$

The data of radioactivity uptake are fitted with the function $A_c(t)$ of equation 10. The least squares best fit

values of K_1, K_2, and r are found by using a program for metric minimization (19) on a CDC 6600 digital computer. Mathematically, the sum of squares of differences (weighted according to the reciprocal of the radioactivity uptake) between the data and $A_c(t)$ is considered as a function F of vector (K_1, K_2, r), and a particular vector point at which F has a minimum is found by the method of steepest descent. Arithmetically, in essence, the gradient of F at a starting point is computed first. Next, a point is located in the direction down the gradient and at a small distance from the starting point. Then the gradient of F at the second point is computed. This leads to a third point. In this fashion, a series of points are successively searched until a point is reached where the gradient of F is zero, i.e., F has a minimum. The operation may be compared to walking down a hillside in search of a valley. F may have more than one minimum; a start on a wrong hillside may lead to a wrong valley. For this reason, an initial guess of (K_1, K_2, r) obtained from graphical analysis of the uptake data serves as the starting point of the search. In the graphical analysis, the limiting slope, K_2, is guessed first. Then the uptake is subtracted from $K_1 + K_2t$, and the difference (K_1e^{-rt}) is plotted against time on a semilog paper to yield K_1 and r.

Following the curve fitting, the fractional rates are calculated according to equations 13-15. The amino acid influx (I) is calculated as $k_{12}N_1(0)$ where $N_1(0)$ is the amount of the amino acid in question initially given to the extracellular fluid. The turnover rate of the intracellular

pool is equal to the sum of all fluxes entering the pool. Accordingly, the influx (I) underestimates this turnover rate by a fraction contributed from biosynthesis and reutilization, and I/r underestimates the intracellular pool size (N_2) by the same endogenous fraction. I/rW underestimates the intracellular concentration of the amino acid. The intracellular amino acid is assumed to be uniformly distributed throughout the cell water. The quotient (Q), flux ratio (influx/efflux) divided by concentration ratio (extracellular/intracellular), is given by

$$Q = (I/k_{21}N_2)/(C_e/C_i)$$

$$= (I/k_{21}C_iW)/(C_e/C_i)$$

$$= I/k_{21}C_eW$$

where C_e and C_i are extracellular and intracellular concentrations of the amino acid, respectively.

D. RESULTS

1. Fitness of Compartmental Model to Data

Figs. 2-5 present individual measurements of the cellular uptake of radioactivity and the least squares best fit form of $A_c(t)$ in equation 10. The fit was generally satisfactory.

Fig. 2 shows the fit in an experiment in which cells were incubated with histidine-imidazole-2-^{14}C in Gey's BSS in duplicate series. One set of pellets of harvested cells was digested for assay of cellular radioactivity; the other

FIGURE 2

Measured radioactivity content in whole cells (open circles), cold TCA extract (triangles), hot TCA extract (closed circles), and TCA precipitate (boxes) upon incubating 1.0×10^8 nucleated dog bone marrow cells in Gey's BSS with L-histidine-imidazole-2-^{14}C at 0.077 mM (SA 3.7 dpm/$\mu\mu$mole). The upper curve represents the best fit form of equation 10 to the radioactivity content in whole cells. The middle curve $\left[A_2(t)\right]$ and the lower curve $\left[A_3(t)\right]$ represent the radioactivity content in compartments II and III, respectively, calculated by application of the model to the radioactivity content in whole cells.

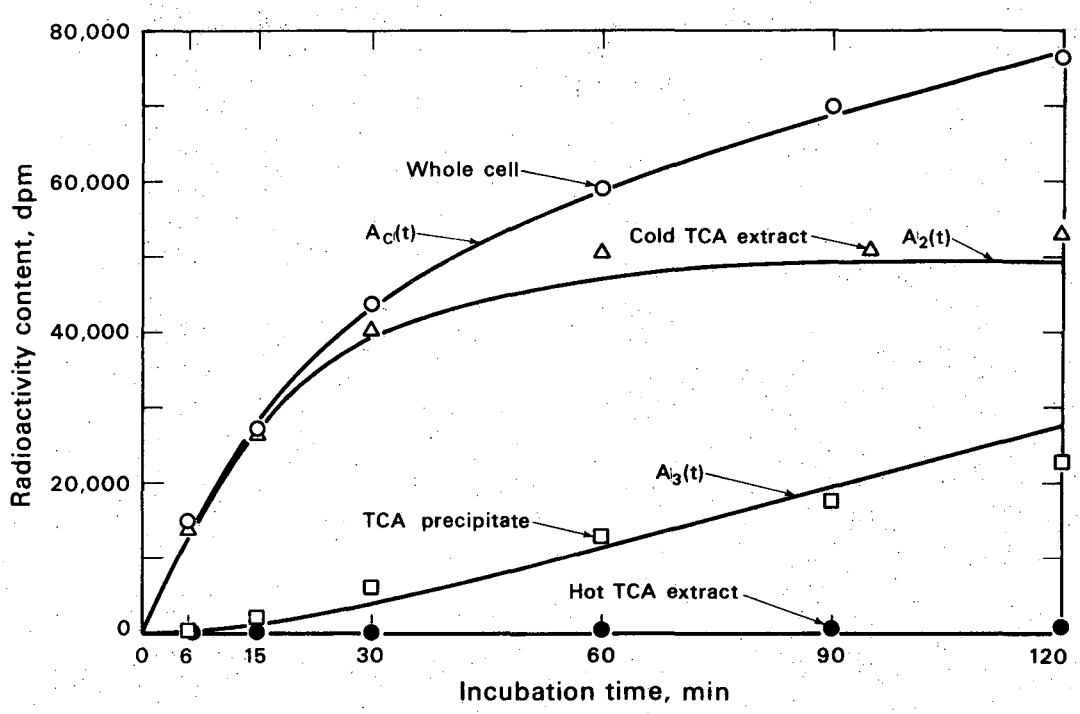
80,000
60,000
40,000
20,000
0
Radioactivity content, dpm
Whole cell
$A_C(t)$
Cold TCA extract
$A_2(t)$
$A_3(t)$
TCA precipitate
Hot TCA extract
0
6
15
30
60
90
120
Incubation time, min

Fig. 2

FIGURE 3

Accumulation of radioactivity inside 7.2×10^7 nucleated dog bone marrow cells incubated in BME with both L-histidine-imidazole-2,5-^{3}H at 0.052 mM (SA 18 dpm/μμmole) and L-serine-3-^{14}C at 0.043 mM (SA 4.2 dpm/μμmole). The measured cellular activity of ^{3}H is shown as circles and that of ^{14}C as triangles. The best fit form of equation 10 to each set of data is represented by the corresponding curves.

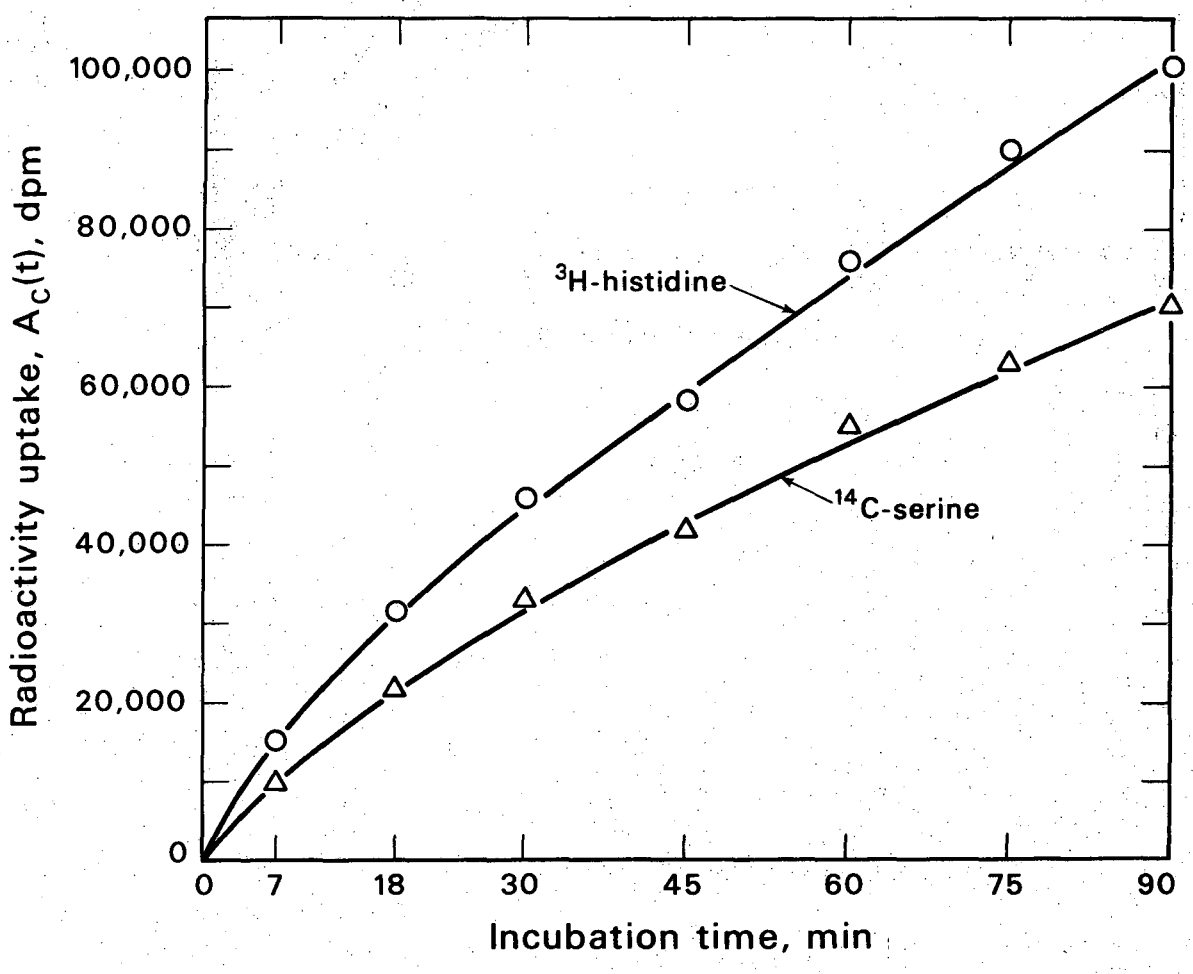
100,000
80,000
60,000
40,000
20,000
0
Radioactivity uptake, $A_C(t)$, dpm
^{3}H-histidine
^{14}C-serine
0
7
18
30
45
60
75
90
Incubation time, min

Fig. 3

FIGURE 4

Accumulation of radioactivity inside 3.6×10^7 nucleated dog bone marrow cells incubated in BME with both L-histidine-2,5-^{3}H at 0.054 mM (SA 18 dpm/$\mu\mu$mole) and L-tryptophan-methylene-^{14}C at 0.024 mM (SA 21 dpm/$\mu\mu$mole). The measured cellular activity of ^{3}H is shown as circles and that of ^{14}C as triangles. The best fit form of equation 10 to each set of data is represented by the corresponding curves.

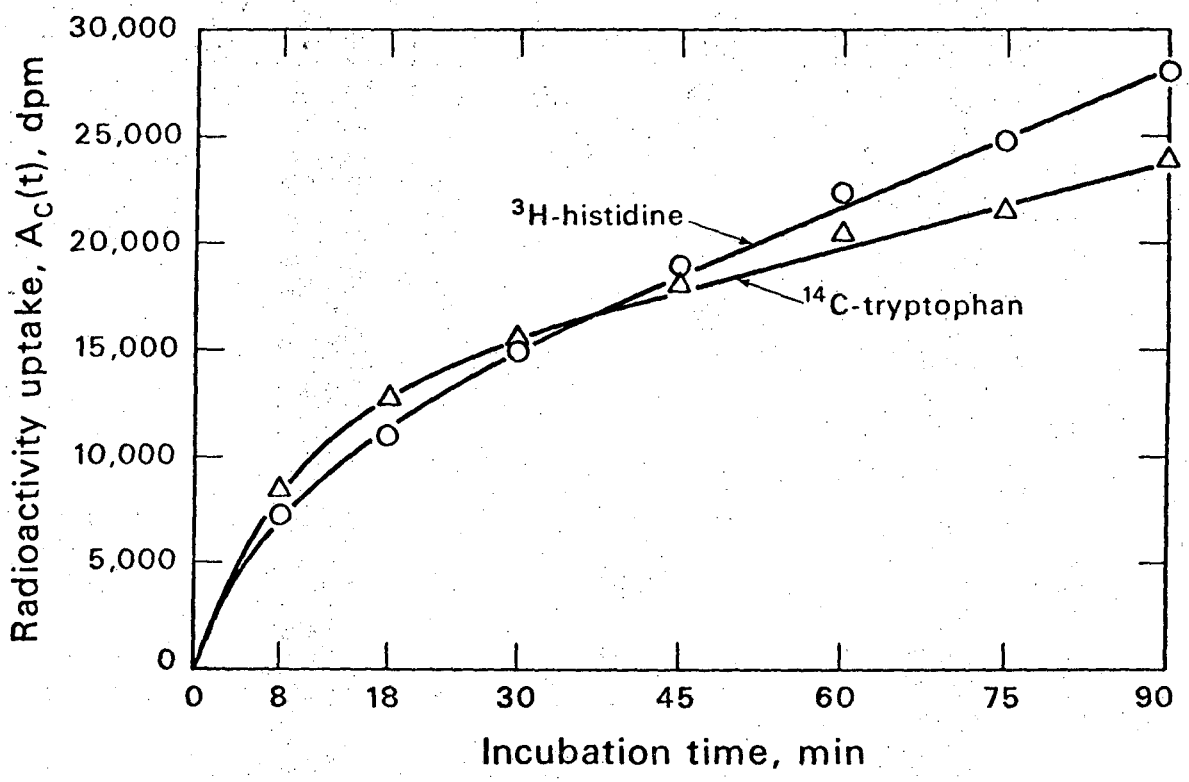
30,000
25,000
20,000
15,000
10,000
5,000
0
Radioactivity uptake, $A_C(t)$, dpm
^{3}H-histidine
^{14}C-tryptophan
0
8
18
30
45
60
75
90
Incubation time, min

Fig. 4

FIGURE 5

Accumulation of radioactivity inside 4.4×10^7 nucleated dog bone marrow cells incubated in BME with both L-histidine-imidazole-2-^{14}C at 0.056 mM (SA 9.7 dpm/μμmole) and L-methionine-methyl-^{3}H at 0.050 mM (SA 26 dpm/μμmole). The measured cellular activity of ^{14}C is shown as circles and that of ^{3}H as triangles. The best fit form of equation 10 to each set of data is represented by the corresponding curves.

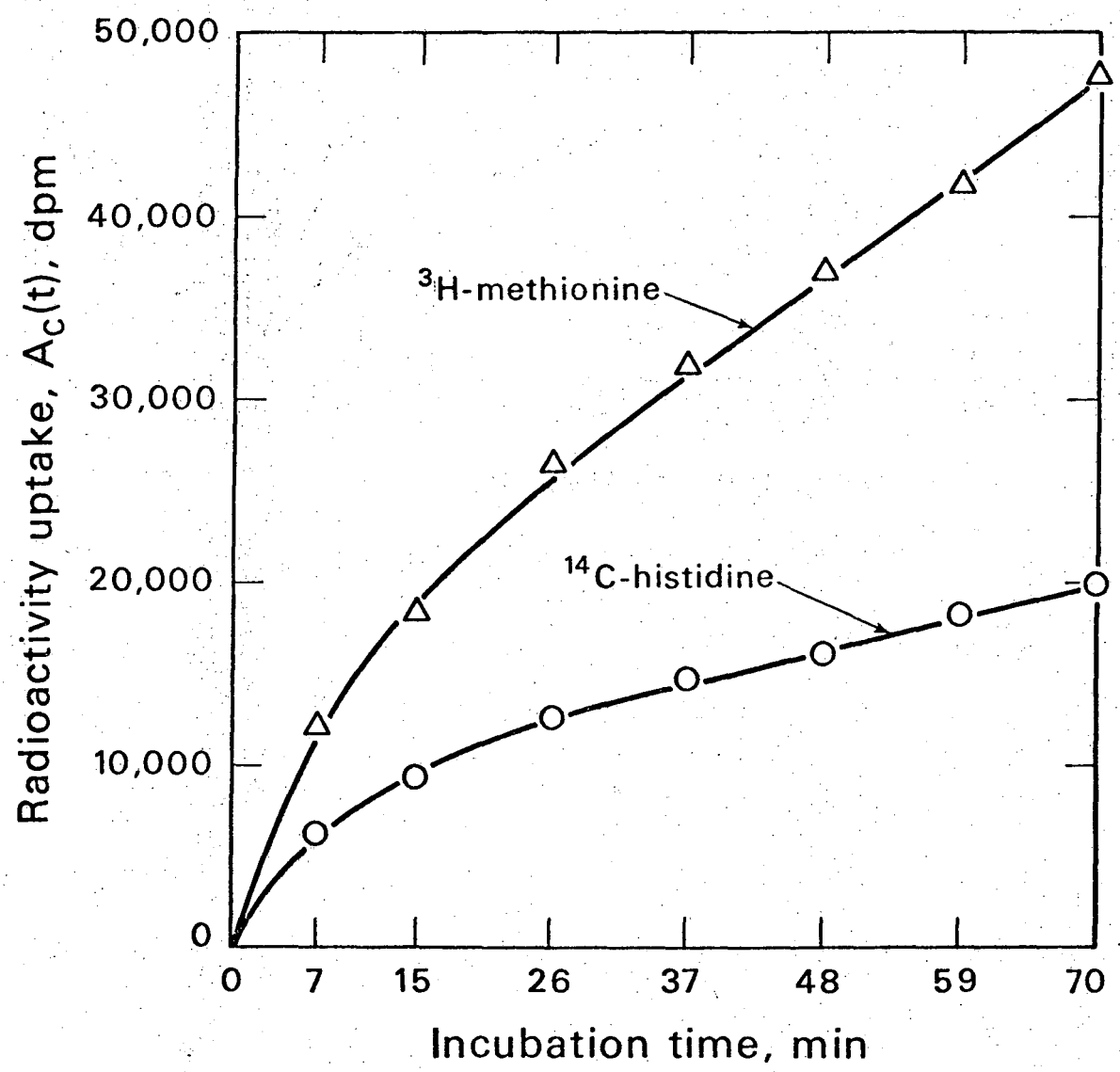
50,000
40,000
30,000
20,000
10,000
0
Radioactivity uptake, $A_c(t)$, dpm
^{3}H-methionine
^{14}C-histidine
0
7
15
26
37
48
59
70
Incubation time, min

Fig. 5

set was subjected to extraction with TCA as described under MATERIALS AND METHODS. From the results of fitting equation 10 to the whole cell radioactivity, the radioactivity in compartments II and III was calculated according to equations 8 and 9, respectively. The measured radioactivity in the cold TCA extract and that in the TCA precipitate corresponded roughly to those calculated to be in compartments II and III of the model, respectively.

2. Kinetics of Intracellular Amino Acid Pools

To study kinetics under relatively physiologic conditions, cells were incubated in BME; when Gey's BSS was used, histidine was the only amino acid given to the culture. In all experiments with serine-3-^{14}C or tryptophan-methylene-^{14}C or methionine-methyl-^{3}H, the culture received the said labeled amino acid together with histidine-imidazole-2,5-^{3}H or histidine-imidazole-2-^{14}C; Figs. 3-5 show the uptake of the individual radioisotopes and the corresponding best fit functions in such double labeling experiments. In experiments using BME, histidine-imidazole-2,5-^{3}H (5 experiments) and histidine-imidazole-2-^{14}C (3 experiments) gave results not significantly different from each other at 20% level; accordingly, these results were combined.

In four experiments, the intracellular water content of the dog bone marrow nucleated cells was estimated to be $W = 14 (\pm 2, SEM) \times 10^{-11}$ ml/cell. For reasons given in MATERIALS AND METHODS, this was felt to be an overestimate.

TABLE I summarizes the incubation conditions and the

TABLE I

Analysis of the Uptake of Labeled Amino Acids by Dog Bone Marrow Nucleated Cells.*

Medium	Gey's Balanced Salt Solution	Basal Medium of Eagle			
Labeled L-amino acid	Histidine-imidazole-2-^{14}C	Histidine-imidazole-2-^{14}C or -2,5-^{3}H	Serine-3-^{14}C	Tryptophan-methylene-^{14}C	Methionine-methyl-^{3}H
Number of experiments	3	8	3	2	3
Extracellular concentration (C_e) mM	0.052	0.052-0.056	0.040-0.043	0.024-0.025	0.050
Fractional turnover rate of intracellular pool (r) min^{-1}	0.161 ± 0.004	0.12 ± 0.01	0.08 ± 0.02	0.11 ± 0.02	0.16 ± 0.03
Influx (I) x 10^8 mμmole/min per cell	2.59 ± 0.06	0.26 ± 0.02	0.55 ± 0.08	0.24 ± 0.02	0.23 ± 0.02
Fractional efflux (k_{21}) min^{-1}	0.152 ± 0.004	0.10 ± 0.01	0.05 ± 0.01	0.11 ± 0.02	0.13 ± 0.02
Fractional metabolic flux (k_{23}) min^{-1}	0.009 ± 0.000	0.024 ± 0.003	0.028 ± 0.006	0.006 ± 0.005	0.033 ± 0.004
Intracellular pool size # (I/r) x 10^8 mμmole/cell	16.1 ± 0.4	2.3 ± 0.3	8 ± 2	2.3 ± 0.6	1.5 ± 0.2
Intracellular concentration # (I/rW) mM	1.15	0.16	0.57	0.16	0.11
Flux ratio/concentration ratio ** # ($I/k_{21}C_eW$)	23	3	19	7	3

* Results, shown below the double line, are means and standard errors of the means.

\# These results were calculated as the quantities in parentheses and were underestimates (see text).

**The quotient of (influx/efflux) divided by (extracellular concentration/intracellular concentration).

results of compartmental analysis. It shows the following: (i) The calculated intracellular concentrations of the four amino acids were higher than their corresponding external concentrations, i.e., the distribution ratios were all greater than unity. (ii) They were all actively transported into these cells, because the quotients of flux ratio (influx/efflux) divided by concentration ratio (extracellular/intracellular) were all greater than unity. (iii) When BME was used as the incubation medium as compared to when Gey's BSS was used at similar histidine concentrations, histidine had slower entry ($p < 0.0005$) and in spite of its smaller "intracellular pool size" (I/r, $p < 0.0005$) had smaller fractional efflux ($p < 0.025$). This finding was an evidence of competition for cell membrane transport between histidine and some other amino acids present in BME. (iv) Even at external concentrations lower than those used with histidine and methionine, serine had faster entry and greater "intracellular pool size" (I/r) than histidine ($p < 0.005$) and methionine ($p < 0.025$). Thus serine was concentrated more readily and to greater extents than histidine and methionine. (v) Judged from the estimate of intracellular metabolism ($k_{23}I/r$), tryptophan appeared to be the least extensively metabolized of these four amino acids; and serine, the most so. (vi) The rate of exit of the amino acid from the cell much exceeded its rate of metabolism within the cell as might be seen from the ratio, k_{21}/k_{23}. With BME as the medium, the average ratios of "exit" to "metabolism", $\langle k_{21}/k_{23} \rangle$, were 11 for tryptophan, 4 for

histidine and methionine, and 2 for serine. (vii) With BME as the medium, the turnover halftime of the intracellular pool (0.693/r) of histidine was 6.0 ± 0.7 (SEM) min; those of the other three amino acids were of the same order of magnitude as that of histidine.

3. Incorporation of the Formimino Carbon of Extracellular FIGLU and the Imidazole-2 Carbon of Histidine into DNA Thymine.

Specific activity of DNA thymine isolated from cells incubated in Gey's BSS with FIGLU-$^{14}C_1$ was determined. Similar experiments were performed with cells incubated with histidine-imidazole-2-^{14}C, which enters the cell as histidine but is metabolized intracellularly to FIGLU-$^{14}C_1$. TABLE II summarizes these experiments performed in the presence and absence of methotrexate at 10^{-4} and 10^{-6} M concentrations. The DNA thymine specific activity in experiments with histidine-imidazole-2-^{14}C was approximately four times as great as that in experiments with FIGLU-$^{14}C_1$ administered at comparable concentrations and specific activities. Addition of methotrexate diminished the DNA thymine ^{14}C specific activity when either histidine-imidazole-2-^{14}C or FIGLU-$^{14}C_1$ was used as the $^{14}C_1$ donor.

Data in TABLE III suggest that the differences in DNA thymine ^{14}C specific activity when histidine-imidazole-2-^{14}C or FIGLU-$^{14}C_1$ was used as the $^{14}C_1$ donor were related to marked differences in cell membrane transport of these two substances. Without prior equilibration in the presence of FIGLU, cells were incubated in Gey's BSS with FIGLU-$^{14}C_1$ at

TABLE II

Incorporation of ^{14}C from L-Histidine-Imidazole-2-^{14}C (HIS-$^{14}C_1$) and α-N-Formimino-L-Glutamate-Formimino-^{14}C (FIGLU-$^{14}C_1$) into DNA Thymine in Dog Bone Marrow Cells in the Presence and Absence of Methotrexate

	Units	Exp. 1		Exp. 2	
Incubation time	hour	1		2	
Concentration of methotrexate when present	M	10^{-4}		10^{-6}	
		HIS-$^{14}C_1$	FIGLU-$^{14}C_1$	HIS-$^{14}C_1$	FIGLU-$^{14}C_1$
Concentration	mM	1	1	0.075	1
Specific activity (SA)	μC/μmole	1.67	1.67	1.67	1.67
SA of DNA thymine					
without methotrexate	dpm/umole	444	119	81	16
with methotrexate	dpm/umole	bgd*	bgd*	49	9

*Sample counting rate not significantly different from the background counting rate.

TABLE III

Radioactivity Uptake by 4.4×10^7 Dog Bone Marrow Nucleated Cells Incubated in Gey's BSS with L-Histidine-Imidazole-2-^{14}C (HIS-$^{14}C_1$) or α-N-Formimino-L-Glutamate-Formimino-^{14}C (FIGLU-$^{14}C_1$) or α-N-Formyl-L-Glutamate-Formyl-^{14}C (FOGLU-$^{14}C_1$)

	Units	HIS-$^{14}C_1$	FIGLU-$^{14}C_1$	FOGLU-$^{14}C_1$
Concentration	mM	0.075	0.075	0.075
Specific activity	dpm/uumole	5.0	5.4	5.4
Radioactivity uptake				
at 1 min	dpm	4,420	40	20
5 min	dpm	16,400	30	50
15 min	dpm	35,500	260	280
30 min	dpm	48,600	320	330

0.075 mM concentration. The intracellular concentration of FIGLU arising from the extracellular FIGLU after 30 min of incubation was calculated to be only 0.010 mM $[A_c(30)N_1(0)/A_1(0)W]$. Similar experiments with α-N-formylglutamic acid (FOGLU) produced similar results while cellular uptake of ^{14}C from histidine-imidazole-2-^{14}C was over 100 times as great as that from FIGLU-$^{14}C_1$.

4. Apparent Kinetics of Histidine Entry and Exodus

The time course of ^{14}C accumulation within cells incubated in the presence of histidine-imidazole-2-^{14}C in Gey's BSS at various histidine concentrations was analyzed according to the compartmental model presented in Fig. 1. As shown in TABLE IV, the fractional influx of histidine decreased with its increasing extracellular concentration, but its fractional efflux stayed essentially unchanged with its rising intracellular level. Thus the efflux of histidine was approximately linear with respect to its intracellular concentration in the range studied. The fractional turnover rate of the intracellular pool was nearly entirely accounted for by the fractional efflux and was nearly independent of intracellular histidine concentration in the range studied.

By using an appropriate minimization program (19), the histidine influx $[k_{12}N_1(0)]$ at various extracellular histidine concentrations was fitted with the function in equation 3. It was assumed that histidine entered the cell by both an apparent diffusion process and a facilitated process. Weighting the squares of deviation of the influx from equation

TABLE IV

Results of Compartmental Analysis of Intracellular Accumulation of Radioactivity in Dog Bone Marrow Nucleated Cells Incubated in 5 ml Gey's BSS with L-Histidine-Imidazole-2-^{14}C at Various L-Histidine Concentrations.

Extracellular concentration of histidine	Fractional rate of entry	Fractional rate of exit	Fractional turnover rate of intracellular pool	Intracellular pool size	Intracellular concentration of histidine*
(S)	$(k_{12}) \times 10^{12}$	(k_{21})	(r)	$(I/r) \times 10^{8}$	(I/rW)
mM	min^{-1} per cell	min^{-1}	min^{-1}	mμmole per cell	mM
0.052	102	0.15	0.16	17	1.2
0.052	96	0.15	0.16	16	1.1
0.052	103	0.16	0.17	16	1.1
0.102	70	0.14	0.15	24	1.7
0.102	85	0.17	0.18	24	1.7
0.502	35	0.15	0.16	54	3.9
0.504	47	0.17	0.18	67	4.8
1.00	29	0.17	0.18	81	5.8
1.00	31	0.17	0.18	87	6.2
1.50	25	0.17	0.17	110	7.9
2.00	20	0.14	0.14	139	9.9
2.00	22	0.16	0.17	132	9.4
3.00	15	0.13	0.13	173	12
4.00	14	0.16	0.16	179	13
4.00	15	0.16	0.17	183	13

* Calculated assuming that intracellular water was 14×10^{-11} ml/cell (see text for results of measurements of intracellular water space).

3 according to the reciprocal of the influx, the optimization gave the following estimates of kinetic constants for histidine entry:

Apparent diffusion constant (k_d) = 4.2 x 10^{-8} mμmole/min per cell per mM.

Apparent Michaelis constant (K_m) = 0.28 mM.

Limiting flux of the facilitated component (V_{max}) = 14 x 10^{-8} mμmole/min per cell.

The fit of the influx to equation 3 with above optimized parameters is shown in Fig. 6. The fit was felt to be satisfactory. At low histidine concentrations in the physiologic range, the facilitated component $\left[V_{max}S(S + K_m)^{-1}\right]$ predominated.

Histidine was actively transported into the cell. Calculated quotients ($Q = I/k_{21}C_eW$) of the flux ratio (influx/efflux) divided by the concentration ratio (extracellular/intracellular) were much greater than unity. The distribution ratios were very high. The intracellular concentration was about 20 times as great as the extracellular concentration when the latter was 0.052 mM.

E. DISCUSSION

1. Validity of the Compartmental Model

The satisfactory fit of equation 10 to the cellular radioactivity data supports the validity of the compartmental model used. If a significant amount of the radioactivity is translocated in the cell from the labeled amino acid to those substances that leave the cell at significant rates, the

FIGURE 6

Rate of L-histidine entry into cells as a function of extracellular L-histidine concentration (S) in dog bone marrow nucleated cells incubated in Gey's BSS in the presence of L-histidine-imidazole-2-^{14}C. Circles represent the calculated rate of entry based on fitting equation 10 to data of ^{14}C accumulation inside cells. The upper curve represents the best fit form of equation 3 to the calculated rate of entry. The lower two curves represent the two components of the upper curve showing the predominance of the saturable component $\left[V_{max}S(S + K_m)^{-1}\right]$ over the nonsaturable component (k_dS) at physiologic concentrations.

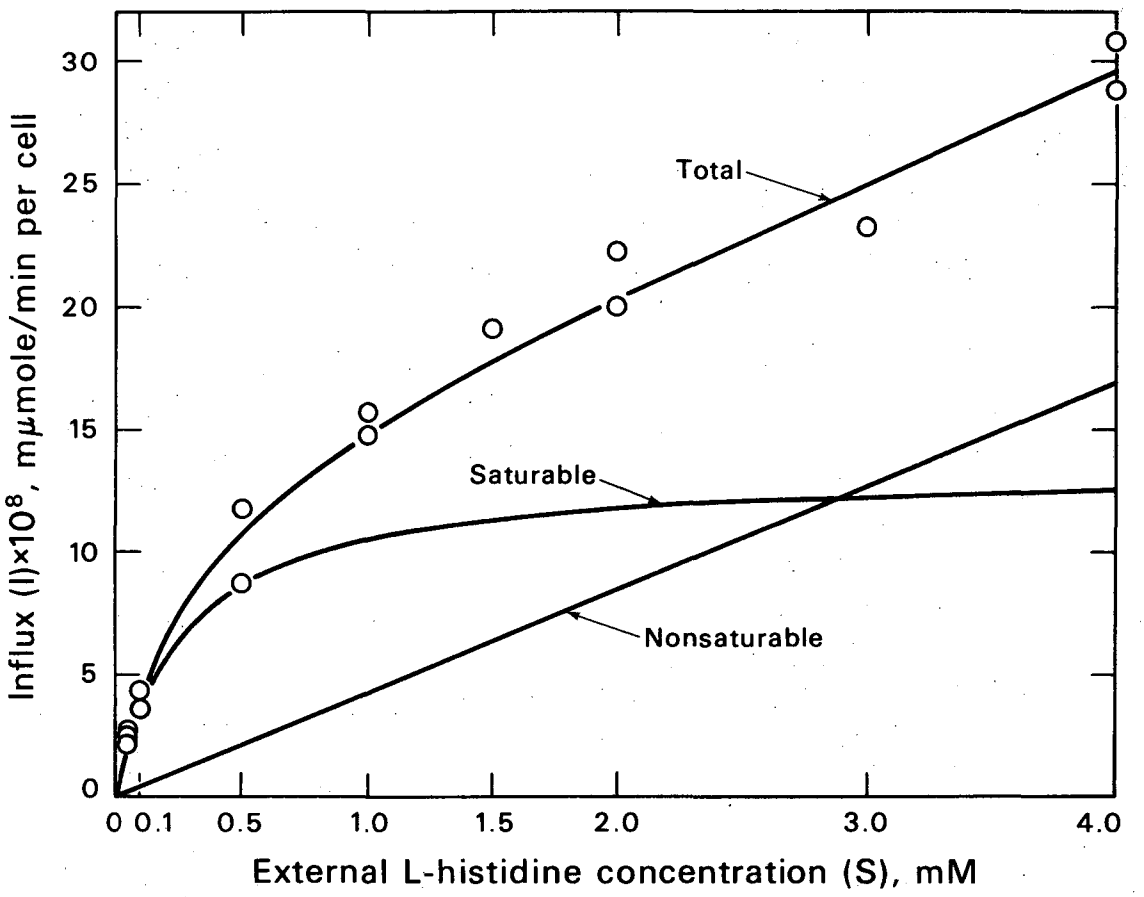
Influx (I)×10⁸, mμmole/min per cell
30
25
20
15
10
5
0
Total
Saturable
Nonsaturable
0 0.1 0.5 1.0 1.5 2.0 3.0 4.0
External L-histidine concentration (S), mM

Fig. 6

model would be a poor approximation to reality. The present study has not excluded such a possibility. Further, different kinds of marrow nucleated cells might have different fractional rates for a given amino acid flux; thus, the present results represent average values for the mixed nucleated cell population of dog bone marrow.

The results of fractionation of cellular radioactivity by TCA extraction (Fig. 2) indicate the following: (i) The intracellular histidine of compartment II is acid-soluble. (ii) Without exogenous supply of amino acids other than histidine, the cellular radioactivity continues to increase even during the second hour. This is in accord with the idea of intracellular reutilization of amino acids in protein synthesis (11). (iii) During the period of incubation, the rate of possible intracellular recycling of labeled histidine from proteins to compartment II is negligible compared to its influx from the extracellular pool.

2. Turnover of the Intracellular Amino Acid Pool

The fractional turnover rate of the intracellular pool of a given amino acid may differ from one tissue to another. Further, it may depend upon the extracellular concentration of other amino acids present as well as upon that of the one under consideration. This is shown to be the case for histidine in the present results.

In radiorespirometric studies, the appearance of $^{14}CO_2$ in the breath following the administration of ^{14}C-labeled amino acids has been used as a measure of *in vivo* metabolic

processes (20). It is clear that such measurements are influenced by a variety of "nonmetabolic" processes, such as the transport of the labeled amino acid across the cell membrane and the turnover of the bicarbonate pool containing the ^{14}C label on carbon dioxide (21). The magnitude of the turnover time of the intracellular amino acid pool measured in the present in vitro study suggests that the rate of equilibration between the extracellular and intracellular pools of a ^{14}C-amino acid constitutes a significant "rate-limiting step" in the production of labeled metabolites, such as $^{14}CO_2$.

3. Transport of FIGLU and FOGLU

The calculated concentration ratios (intracellular/extracellular) of FIGLU and FOGLU after 30 min of incubation are both much smaller than unity indicating either one or both of the following possibilities: (i) At the concentrations used, their net entry is so slow that their intracellular concentrations can not closely approach equilibrium concentrations during the experiment. (ii) Their equilibrium intracellular concentrations are far below their extracellular concentrations (cf. equation 8 for very small R/r).

At neutral pH, glutamine and glutamate-γ-methyl ester (both virtually devoid of net charges) exhibit evidences of active absorptive transport across intestinal walls while glutamate (with net negative charges) failed to do so (22). Judged by such findings of others (22), the poor uptake of FIGLU and FOGLU in the present experiments may be related to their net charges at neutral pH [roughly a single net negative

charge on FIGLU in accordance with its pK_a values (23) and two net negative charges on FOGLU].

4. Transport of Histidine

The fit shown in Fig. 6 supports the notion that histidine entry into the nucleated cells of dog bone marrow can be described as a combination of an apparent first order process and a facilitated process. Similar behavior has been observed in human erythrocytes (3), rat diaphragms (5), guinea pig lymph node cells (24), and Ehrlich ascites tumor cells (8) for certain other neutral amino acids. The present demonstration of active entry of histidine into the nucleated cells of dog bone marrow clearly indicates that facilitating system or systems do participate in the transport of histidine into these cells. Simple diffusion alone can not bring about such uphill transport against concentration gradient. The K_m presently estimated for histidine entry is remarkably similar to those reported for the entry of lysine and phenylalanine into Ehrlich ascites tumor cells (9).

A facilitated component of a flux approaches first order kinetics ($V_{max}S/K_m$) at concentrations much lower than the K_m. The following argument suggests that the apparent first order component of histidine influx in the present experiments with Gey's BSS does not represent a single simple diffusion process but rather a summation of simple diffusion and one or more facilitated processes with high K_m values. If the apparent diffusion constant for histidine entry is indeed a simple diffusion constant, it would then be the simple diffusion

constant for histidine exodus also. The fractional rate of histidine exodus (k_{21}) is an upper bound of the fractional rate of histidine exodus by simple diffusion alone, i.e., an upper bound of the simple diffusion constant for histidine exodus expressed in min^{-1}. By considering an amount of extracellular fluid equal to cell water in volume, the apparent diffusion constant can be expressed as k_d/W in min^{-1} relative to the same frame of reference as that for k_{21}. As given in RESULTS,

$$k_d = 4.2 \times 10^{-11} \ \mu mole/min \text{ per cell per mM}$$

$$W = 14 \times 10^{-11} \ ml/cell$$

Thus $k_d/W = 0.30 \ min^{-1}$, a value much greater than any k_{21} in the present results.

In the present experiments with BME, the fractional efflux of histidine is 0.10 ± 0.01 (SEM) min^{-1} (TABLE I). This fractional efflux is substantially smaller than that of 0.15 ± 0.01 (SEM) min^{-1} obtained in experiments with Gey's BSS (TABLE I). Thus a substantial portion of histidine exodus from dog bone marrow nucleated cells when they are incubated in Gey's BSS appears to be susceptible to analogue inhibition and hence facilitated. Consequently, the apparent first order kinetics of histidine exodus observed at intracellular histidine concentrations in the range up to 13 mM (TABLE IV) would indicate a K_m for histidine exodus much greater than that of 0.28 mM estimated for its entry. This conclusion finds support in a recent study in Ehrlich ascites tumor cells by

Christensen and Handlogten (9). The reported K_m values for exodus of the four amino acids in their study exceed the corresponding values for entry by more than tenfold.

F. SUMMARY

Dog bone marrow nucleated cells were incubated in media containing labeled L-amino acids, and the cellular accumulation of radioactivity as a function of time was measured and analyzed according to a three-compartment model.

The turnover halftime of intracellular histidine arising from extracellular sources was 6.0 $\pm$ 0.7 (SEM) min. Similar results were obtained for serine, tryptophan, and methionine. Exodus of the amino acid accounted for a major portion of this turnover. These results indicate that subsequent to the administration of labeled amino acids the rate of equilibration between the extracellular and intracellular pools constitutes an important "rate-limiting step" in the production of labeled metabolites. α-N-formiminoglutamate added to the culture was inferior to histidine as a source of monocarbon fragments because the former accumulated inside the cell much more slowly than the latter.

Histidine, serine, tryptophan, and methionine were actively transported into the cell. At the extracellular histidine concentrations used (up to 4 mM), histidine appeared to enter by both a facilitated process with an apparent Michaelis constant of 0.28 mM and a limiting flux of 14 x 10^{-8} mμmole/min per cell, and an apparent first order process not solely accountable by simple diffusion. At physiologic extracellular

histidine concentrations, histidine entry by the former process prevailed over that by the latter process. Exodus of histidine appeared to be substantially facilitated, but exhibited an apparent first order process at intracellular histidine concentrations in the range studied (up to 13 mM), and thus appeared to have an apparent Michaelis constant much greater than that of histidine entry.

III. EFFECTS OF L-METHIONINE ON THE KINETICS OF INTRACELLULAR POOLS OF L-HISTIDINE AND L-SERINE

A. INTRODUCTION

Methionine is known to compete with other neutral amino acids for cell membrane transport (25). The following work evaluates such a competition in the nucleated cells of dog bone marrow.

B. MATERIALS AND METHODS

Details for the method of preparing cell suspension, incubation, assay of cellular radioactivity, and compartmental analysis had been presented in Part II of this paper. Amino acids used were of L form. Basal Medium of Eagle without methionine was obtained from Grand Island Biological. Serine was added to this medium to a concentration of 0.017 or 0.040 mM. The term, BME, in this part of the paper referred to the resultant medium.

Equal portions of the cell suspension were delivered into a series of incubation tubes containing equal volume of Gey's BSS or BME with and without known amounts of methionine. After equilibration at 37°C for 20-30 min, tracer quantities of labeled amino acids were added in equal amounts to all tubes.

C. RESULTS

When methionine was present in the incubation medium containing histidine-imidazole-2-^{14}C, the cellular ^{14}C uptake was depressed as compared to that in similar incubations

in the absence of methionine (Figs. 7 and 8). Inspection of such data led to the conclusion that methionine depressed the initial rate of cellular ^{14}C accumulation and hence the rate of histidine entry into the cell. Furthermore, the methionine concentration required to produce a given depression in histidine influx was higher in the presence of other amino acids (BME, Fig. 8) than in their absence (Gey's BSS, Fig. 7).

In two experiments, cells were incubated in BME in the presence of both histidine-imidazole-2,5-^{3}H and serine-3-^{14}C with and without methionine (3.2 mM) in the medium. Fig. 9 shows the fit of equation 10 to the cellular uptake of radioactivity in such an experiment. Results in TABLE V show that the methionine depressed the influx, the fractional efflux, the fractional metabolic flux, and the fractional turnover rate of the intracellular pool of both histidine and serine. Since it was likely that methionine administration had not raised their intracellular concentrations, their depressed fractional fluxes indicated depressions of the corresponding fluxes.

D. DISCUSSION

The present finding that methionine can depress the entry and exodus of histidine and serine suggests that the transport of these three neutral amino acids in either direction is facilitated to some extents in the nucleated cells of dog bone marrow. This is consistent with previous conclusions that they are all actively transported into the cell and that both entry and exodus of histidine have facilitated components

FIGURE 7

Accumulation of radioactivity inside dog bone marrow nucleated cells incubated in Gey's BSS with L-histidine-imidazole-2-^{14}C at 0.075 mM (SA 1.7 μC/μmole) in the presence (closed circles) and absence (open circles) of 0.075 mM L-methionine.

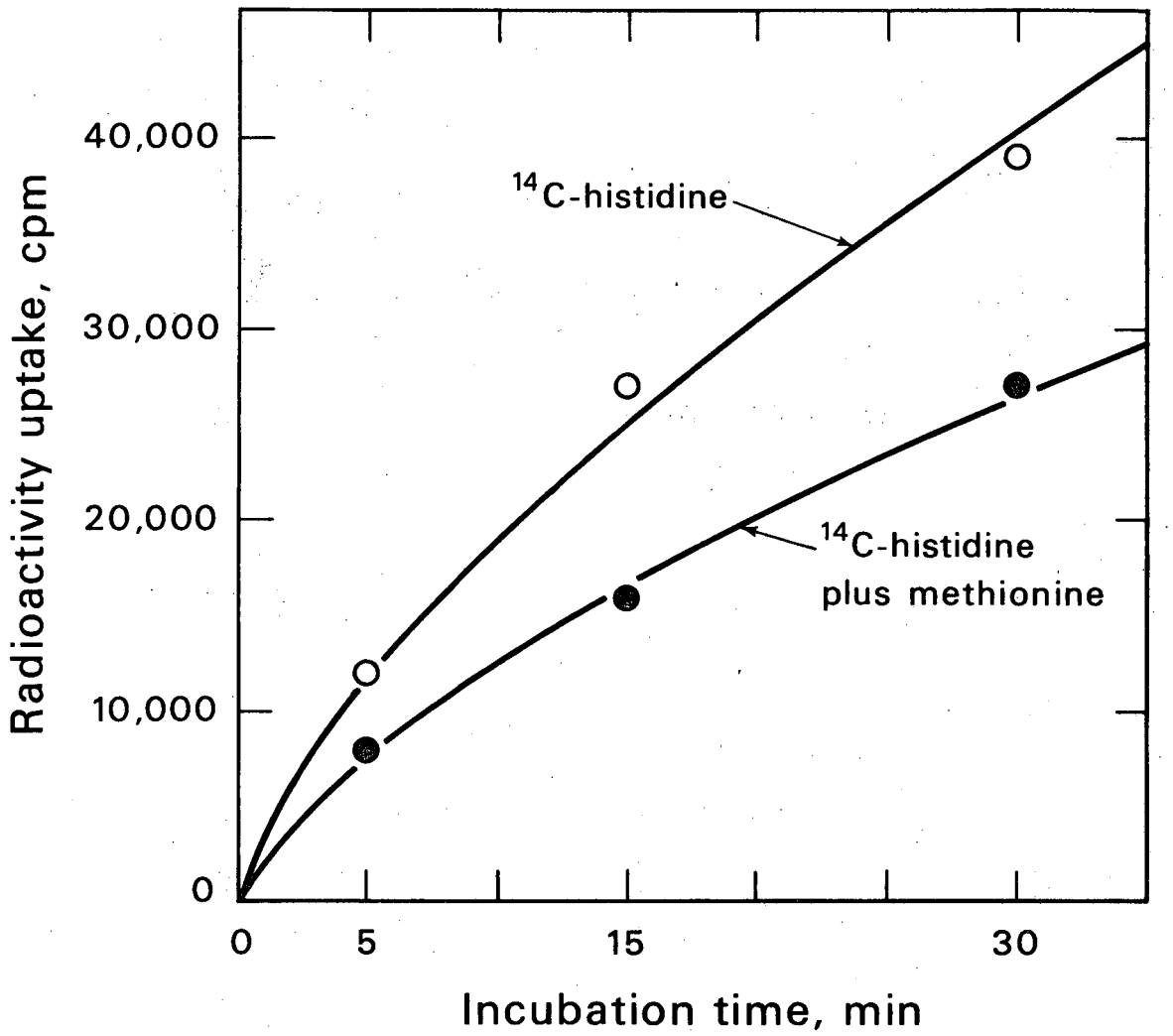
40,000
30,000
20,000
10,000
0
Radioactivity uptake, cpm
^{14}C-histidine
^{14}C-histidine
plus methionine
0
5
15
30
Incubation time, min

Fig. 7

FIGURE 8

Accumulation of radioactivity inside dog bone marrow nucleated cells incubated in BME (i) without L-methionine (open circles), (ii) with 0.213 mM L-methionine (closed circles), and (iii) with 1.07 mM L-methionine (triangles).

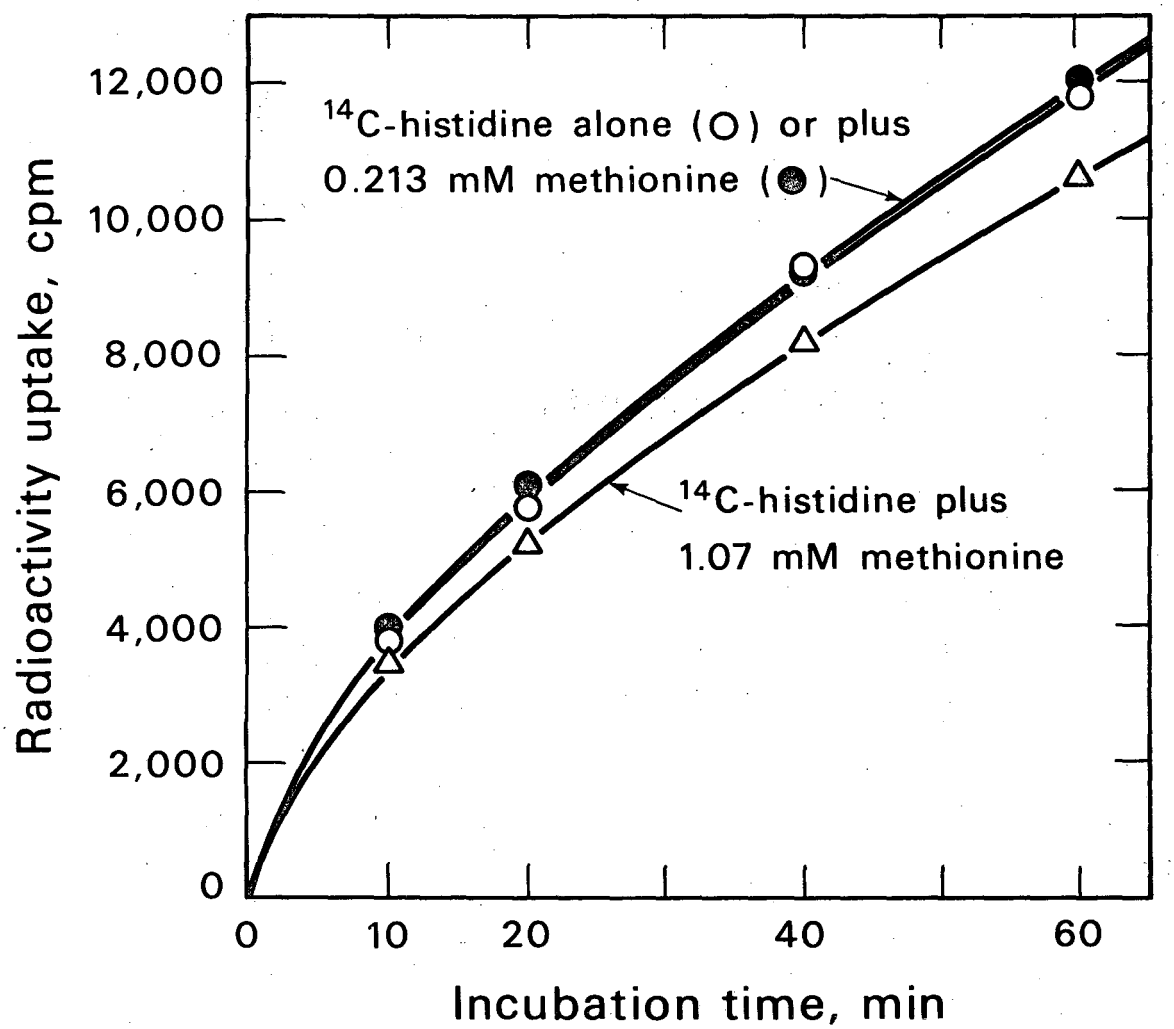
12,000
10,000
8,000
6,000
4,000
2,000
0
Radioactivity uptake, cpm
^{14}C-histidine alone (O) or plus
0.213 mM methionine (●)
^{14}C-histidine plus
1.07 mM methionine
0
10
20
40
60
Incubation time, min

FIGURE 9

Accumulation of radioactivity inside 3.0×10^7 nucleated dog bone marrow cells incubated in BME with both L-histidine-imidazole-2,5-^{3}H at 0.054 mM (SA 42 dpm/$\mu\mu$mole) and L-serine-3-^{14}C at 0.024 mM (SA 20 dpm/$\mu\mu$mole) in the presence and absence of 3.20 mM L-methionine. The measured cellular activity of ^{3}H is shown as open (control) and closed (3.20 mM L-methionine) circles and that of ^{14}C as triangles (control) and boxes (3.20 mM L-methionine). The best fit form of equation 10 to each set of data is represented by the corresponding curves.

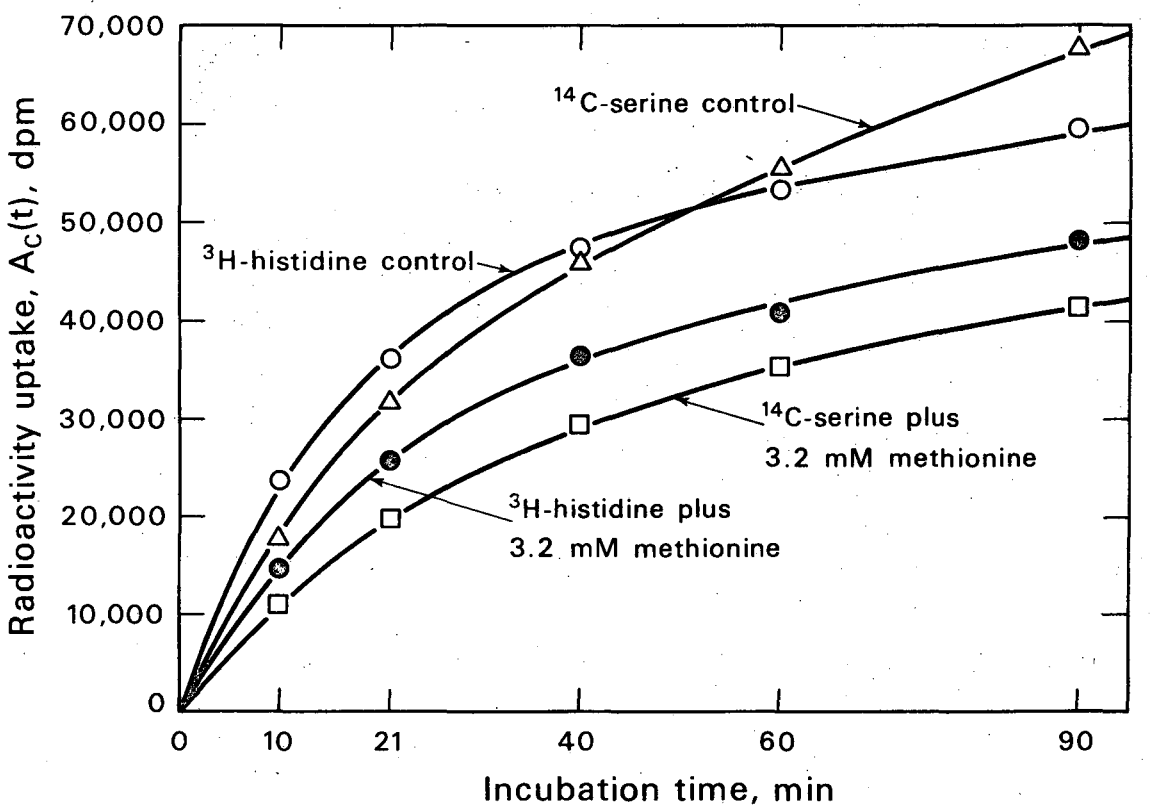
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
Radioactivity uptake, $A_C(t)$, dpm
0
10
21
40
60
90
Incubation time, min
^{14}C-serine control
^{3}H-histidine control
^{14}C-serine plus
3.2 mM methionine
^{3}H-histidine plus
3.2 mM methionine

Fig. 9

TABLE V

Effects of 3.2 mM L-methionine on the Kinetics of the Intracellular Pools of L-histidine and L-serine in the Nucleated Cells of Dog Bone Marrow Incubated in BME

		L-histidine		L-serine	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
Concentration	mM	0.054	0.052	0.024	0.040
Fractional turnover rate of intracellular pool (r)	%*	66	55	75	56
Influx (I)	%	57	69	59	59
Fractional efflux (k_{21})	%	65	55	80	55
Fractional metabolic flux (k_{23})	%	83	55	46	56
"Intracellular pool size" (I/r)	%	86	127	79	105

*The kinetic quantities listed are presented as % of the corresponding control values.

(Part II). The observed extents of depression in experiments with BME (TABLE V) further suggest that facilitated transport represents a substantial component of the transport of histidine and serine in either direction even when the facilitation of their transport is expected to be submaximal in the presence of other competing amino acids (BME).

The extent of depression of histidine influx by methionine in the present results provides another bit of evidence that the k_d estimated for histidine entry (Part II) is not a simple diffusion constant. If the k_d estimated for histidine entry in the virtual absence of other amino acids from the medium (Gey's BSS, Part II) indeed represents a simple diffusion constant, it would also represent the simple diffusion constant for histidine entry in the presence of other amino acids at modest concentrations (BME). Under the circumstances, the simple diffusion component of histidine influx at 0.052-0.054 mM histidine concentrations in BME would amount to 0.22×10^{-8} mμmole/min per cell (k_dS). This component, presumably not susceptible to analog inhibition, would represent about 85% of the total influx (0.26×10^{-8} mμmole/min per cell, TABLE I) incompatible with the depression of the influx to 57 or 69% by methionine (TABLE V).

E. SUMMARY

Analogue inhibition of the transport of L-histidine and L-serine by L-methionine has been demonstrated in dog bone marrow nucleated cells suspended in Basal Medium of Eagle. L-methionine depressed the influx, the efflux, and the

fractional turnover rate of the intracellular pool of both L-histidine and L-serine. These results suggest that substantial portions of both entry and exodus of both L-histidine and L-serine are facilitated even in the presence of other amino acids (Basal Medium of Eagle).

IV. EFFECTS OF INSULIN AND GLUCAGON ON THE TRANSPORT OF L-HISTIDINE

A. INTRODUCTION

The effect of insulin added *in vitro* on the cellular accumulation of natural amino acids has been studied in rat diaphragms (5, 26, 27). In these studies, the concentration ratios (intracellular/extracellular) of glycine, methionine, proline, hydroxyproline, and threonine after 1 or 2 hours of incubation were found to be enhanced by the presence of insulin (about 0.1 - 0.5 U/ml) in the medium. The concentration ratios of other natural amino acids studied were not affected (5, 27, 28). The concentration ratio, however, provides little information concerning the fluxes through the intracellular pool of the amino acid. It is clear that intracellular concentration of the amino acid depends upon all these fluxes. For this reason, the concentration ratio is not an ideal parameter for studying the effect of insulin or other agents on amino acid transport.

Garcia, Williamson, and Cahill have reported that ^{14}C incorporation from ^{14}C-labeled alanine into glucose by perfused rat livers were stimulated by the presence of glucagon in the perfusate (29). It is unclear whether such gluconeogenic effect may arise partly from possible glucagon effect on amino acid transport.

The present study evaluates the effect of insulin and glucagon on histidine transport in the nucleated cells of dog bone marrow by the method previously described in Part

II of this paper.

B. MATERIALS AND METHODS

Stock solutions of insulin (24 U/ml) and glucagon (0.15 mg/ml) were prepared by dissolving crystalline bovine insulin (Sigma Chemical Company, St. Louis, Mo.) and crystalline glucagon (Calbiochem, Los Angeles, Calif.), respectively, in 0.002 N HCl; kept at 4°C for no longer than 1 week; and diluted with Gey's BSS immediately before individual experiments.

Two ml portions of cell suspension were delivered into a series of incubation tubes containing equal volume of Gey's BSS at known concentrations of histidine and insulin or glucagon. Control tubes received no insulin or glucagon. After equilibration of the tube content at 37°C for 20-30 min, tracer quantities of histidine-imidazole-2-^{14}C were added in equal amounts to all tubes. Methods of preparing the cell suspension, incubation, assay of cellular radioactivity, and compartmental analysis had been described in Part II of this paper.

C. RESULTS

TABLE VI summarizes the results of compartmental analysis of the radioactivity uptake by these cells incubated in Gey's BSS in the presence of histidine-imidazole-2-^{14}C with and without added insulin or glucagon. Neither insulin (0.1 U/ml) nor glucagon (1 μg/ml) had substantial effect on the kinetics of histidine at external concentrations used except for a

TABLE VI

Effects of Insulin (0.1 U/ml) or Glucagon (1 μg/ml) on the Kinetics of the Intracellular L-Histidine Pool in the Nucleated Cells of Dog Bone Marrow Suspended in Gey's BSS

		Insulin				Glucagon		
		Exp. 1	Exp. 2	Exp. 3		Exp. 4	Exp. 5	
Concentration	mM	0.052	0.052	0.052		0.102	0.102	
					Mean			Mean
Fractional turnover rate of intracellular pool (r)	%*	96	109	103	103	102	92	97
Influx (I)	%	103	111	104	106	98	96	97
Fractional efflux (k_{21})	%	95	109	103	102	102	93	98
Fractional metabolic flux (k_{23})	%	103	119	112	111	106	87	97
"Intracellular pool size" (I/r)	%	107	102	101	103	96	104	100

*The values listed are presented as % of the corresponding control values.

possible small increase in the fractional metabolic flux when insulin was present in the medium.

D. DISCUSSION

The external histidine concentrations used in the present experiments are quite lower than the K_m of 0.28 mM estimated for histidine entry into these cells (Part II). Any substantial change in K_m or V_{max} for histidine entry upon the administration of insulin and glucagon at pharmacologic concentrations would have substantially altered the histidine influx contrary to the present results since its facilitated component, the major one at the histidine concentration used, would have been so altered. The lack of substantial insulin effect on the K_m of insulin entry into dog bone marrow nucleated cells contrasts with the remarkable fall in K_m for AIB entry into isolated rat diaphragms due to similar insulin administration reported by Akeda and Christensen (5).

In an <u>in vitro</u> study with rabbit bone marrow slices, Necheles (30) has reported that insulin (0.1 U/ml) enhances the incorporation of histidine-imidazole-2-^{14}C into cellular proteins by about 30% of the control values after 1 hour of incubation. In view of the present results (TABLE VI), enhanced transport is an unlikely explanation for the enhanced incorporation in his observation.

Chambers, Georg, and Bass (31) have demonstrated that glucagon administered <u>in vitro</u> as well as <u>in vivo</u> remarkably stimulates the uptake of AIB, an unnatural amino acid, by perfused isolated rat livers. To what extent the known gluconeo-

genic effect of glucagon may depend upon its possible effect on the cell membrane transport of natural amino acids awaits further investigations.

E. SUMMARY

The effect of insulin and glucagon on the kinetics of L-histidine transport has been studied in the nucleated cells of dog bone marrow suspended in Gey's Balanced Salt Solution. Neither insulin (0.1 U/ml) nor glucagon (1 μg/ml) had substantial effect on the influx, the fractional efflux, and the fractional turnover rate of the intracellular pool. A possible small increase in the fractional metabolic flux was found when insulin was present in the medium.

V. EFFECTS OF PYRIDOXAL ON THE KINETICS OF INTRACELLULAR POOLS OF L-HISTIDINE AND L-TRYPTOPHAN IN BONE MARROW CELLS

A. INTRODUCTION

Some evidences for the possible role of pyridoxal in amino acid transport have led Christensen, Riggs, Aspen, and Mothon (32) to propose a hypothesis that pyridoxal might function as a carrier in amino acid transport. The two compounds were supposed to combine to yield a Schiff's base. A complex formed by chelation of a metal ion to the base was thought to be the diffusing unit. From their earlier work (33) prior to the proposal, they knew that the alleged action of pyridoxal would not represent a physiological one. At concentrations below 0.1 mM, pyridoxal rapidly lost its stimulatory effect on glycine accumulation in Ehrlich ascites tumor cells. Subsequently, the hypothesis was abandoned by these investigators themselves for the following reason: As a test of the carrier function of a compound, Christensen (34) proposed that the entry of the compound itself into the cell should be accelerated by the presence of amino acid in the extracellular fluid. To their regret, they found that most of the amino acids tested actually decreased the uptake of pyridoxal by Ehrlich ascites tumor cells (35). The basis for their rejection of the alleged role of pyridoxal as a carrier of amino acids does not appear, to the present author, to be valid. It is quite possible that the hypothetical complex can enter the cell more readily than the amino acid but less readily than the carrier itself.

Two considerations have prompted the present study. (i) It was felt plausible that an alteration in amino acid transport might underlie the alleviation of the clinical syndrome, "pyridoxine-responsive anemia", by massive pyridoxine therapy. A study of pyridoxal effect on amino acid transport in the nucleated cells of bone marrow would seem highly indicated. (ii) Ngo (36) at this laboratory had studied the effect of pharmacological doses of pyridoxine on the appearance of $^{14}CO_2$ in the breath following the administration of L-tryptophan-1-^{14}C, L-histidine-imidazole-2-^{14}C, L-methionine-methyl-^{14}C, and $NaH^{14}CO_3$ to rats. Rats were given the ^{14}C-compound intravenously with and without prior intravenous administration of 100 mg pyridoxine hydrochloride 30 min before the injection of the ^{14}C-compound. He found that the pyridoxine administration resulted in decreased rate of $^{14}CO_2$ excretion in the breath only in rats given L-tryptophan-1-^{14}C. The breath $^{14}CO_2$ production in rats given other ^{14}C-compounds studied was not affected.

B. MATERIALS AND METHODS

1. Materials

Basal Medium of Eagle with L-glutamine but without pyridoxal hydrochloride and phenol red was obtained from Grand Island Biological Company (Grand Island, N.Y.) and referred to simply as BME in this part of the paper. Pyridoxal hydrochloride was obtained from Calbiochem (Los Angeles, Calif.) and kept in the dark until use. Solutions of pyridoxal were prepared immediately before individual experiments.

Equimolar amounts of pyridoxal hydrochloride powder and 1 N NaOH solution were mixed together first. The mixture was then dissolved and diluted in BME to yield desired final concentration of pyridoxal in culture fluid in experiments on cellular uptake of labeled amino acids. In experiments on hemolysis due to ammonium oxalate in isotonic solutions containing varying amounts of pyridoxal, the said mixture was dissolved and diluted to desired final pyridoxal concentrations in distilled water containing ammonium oxalate (80 mM) and NaCl (20 mM).

2. Accumulation of Radioactivity from Labeled Amino Acids by Dog Bone Marrow Cells and Compartmental Analysis of the Radioactivity Uptake as a Function of Incubation Time

Methods used had previously been described in Part II of this paper. Two ml portions of cell suspension were delivered into a series of tubes containing 2 ml BME with and without pyridoxal in the BME. After equilibration at 37°C for 20-30 min, tracer quantities of labeled amino acids were added in equal amounts to all tubes.

3. ^{14}C Accumulation from Labeled Tryptophan by Human Erythrocytes

Erythrocytes were isolated from heparinized venous blood of a healthy young adult, washed once in BME, and resuspended in BME. Portions of the suspension were delivered into a series of tubes containing BME with and without pyridoxal in the BME. After equilibration at 37°C for 15 min, tracer quantity of labeled tryptophan was added to all tubes.

After 2, 5, 10, and 20 min incubation at 37°C, the tube content was passed through a membrane filter (Nalgene Filter Unit, membrane pore size 0.2 μ; Nalgene Labware Cat. No. 120, Rochester, N.Y.) under suction. Cells recovered on the membrane were washed similarly with medium and digested together with the membrane in NCS. The digest was then assayed for radioactivity by liquid scintillation counting. Suction was applied to speed up the filtration since amino acid exchange across the erythrocyte membrane was expected to be very rapid. This suction, however, appeared to have caused some cell breakage.

4. Lysis of Erythrocytes in Ammonium Oxalate Solution

Human erythrocytes were isolated from heparinized venous blood of a normal adult, washed once in 3 volumes of Gey's BSS, and resuspended in 130 volumes of 145 mM NaCl solution. Four ml portions of the suspension were added to duplicated series of tubes containing 10 ml isotonic solutions at equal concentration of ammonium oxalate (80 mM) and NaCl (20 mM) but at varying concentrations of pyridoxal (0-1 mM). The extracellular fluid of the resultant suspension in all tubes was initially equivalent osmotically to 141-142 mM NaCl. After standing at room temperature for 5 min, the tube content was centrifuged at 4°C for 4 min. Optical density of the separated supernatant hemolysate was read at 541 mμ in a Beckman DU Spectrophotometer against distilled water as blank.

5. Osmotic Fragility of Erythrocytes in Hypotonic NaCl Solution

A total of four experiments were performed on erythrocytes from three healthy laboratory workers. In each experiment, erythrocytes were isolated from heparinized venous blood of a subject. After one washing in Gey's BSS, cells were incubated in 100 volumes of Gey's BSS in the absence and presence of pyridoxal in the medium at 37°C for 30 min. Following the incubation, cells were recovered by centrifugation, washed twice in 3 volumes of Gey's BSS, and resuspended in 3 volumes of Gey's BSS. 0.1 ml portions of the suspension of incubated and washed cells were then added to a series of tubes containing 10 ml buffered NaCl solutions at varying effective NaCl concentrations. The buffered NaCl solutions were prepared by a standard technique (37). After standing at room temperature for 45 min, the tube content was centrifuged, and optical density of the separated supernatant fluid was read at 541 mμ against distilled water as blank in a Beckman DU Spectrophotometer.

6. pH of Extracellular Fluid during Incubation

In four separate experiments in which bone marrow cells or erythrocytes were incubated in BME or Gey's BSS in the absence and presence of pyridoxal in the medium, pH of the extracellular fluid from control tubes at the beginning of incubation was in the range 7.4-7.8 as determined in a pH meter (Beckman Zeromatic II). After 30 min incubation, pH rose slightly, but never exceeded the initial value by more

than 0.3 units. Either at the beginning or after 30 min of incubation, pH of the fluid from incubation tubes containing pyridoxal (2.5 mM) agreed with corresponding control values to within 0.3 units.

C. RESULTS

1. Effects of Pyridoxal on Cellular Accumulation of Labeled Histidine and Tryptophan

Dog bone marrow nucleated cells (contaminated by 2-4 erythrocytes for every nucleated cell present) were incubated with histidine-imidazole-2,5-^{3}H and tryptophan-methylene-^{14}C in the absence (control) and presence of pyridoxal (2.5 mM) in BME. Then after one brief (about 1 min) washing in 80 mM ammonium oxalate solution, cells were packed by centrifugation, and the isolated cell pellets were assayed for ^{3}H and ^{14}C contents. The pyridoxal was found to stimulate the radioactivity uptake from both labeled amino acids. However, while the washing in ammonium oxalate solution lysed most of the contaminating erythrocytes from control incubations resulting in slightly pinkish pellets and pinkish supernatant hemolysate, similar washing of cells from incubations in the presence of pyridoxal resulted in pellets as deeply red-colored as before the washing and nearly colorless supernatant wash. The radioactivity uptake as a function of incubation time was analyzed according to the compartmental model shown in Fig. 1 (Part II). Fig. 10 shows the measured radioactivity uptake and the corresponding best fit form of equation 10 from such an experiment. The difference in

FIGURE 10

Accumulation of radioactivity inside 5.2×10^7 nucleated dog bone marrow cells incubated in BME with both L-histidine-imidazole-2,5-^{3}H at 0.052 mM (SA 9.8 dpm/uumole) and L-tryptophan-methylene-^{14}C at 0.024 mM (SA 20 dpm/uumole) in the absence and presence of 2.5 mM pyridoxal. The measured cellular activity of ^{3}H is shown as open (control) and closed (2.5 mM pyridoxal) circles and that of ^{14}C as triangles (control) and boxes (2.5 mM pyridoxal). As explained in the text, the measurement for uptake in the presence of pyridoxal includes radioactivity in significant number of contaminating erythrocytes. The best fit form of equation 10 to each set of data is represented by the corresponding curve.

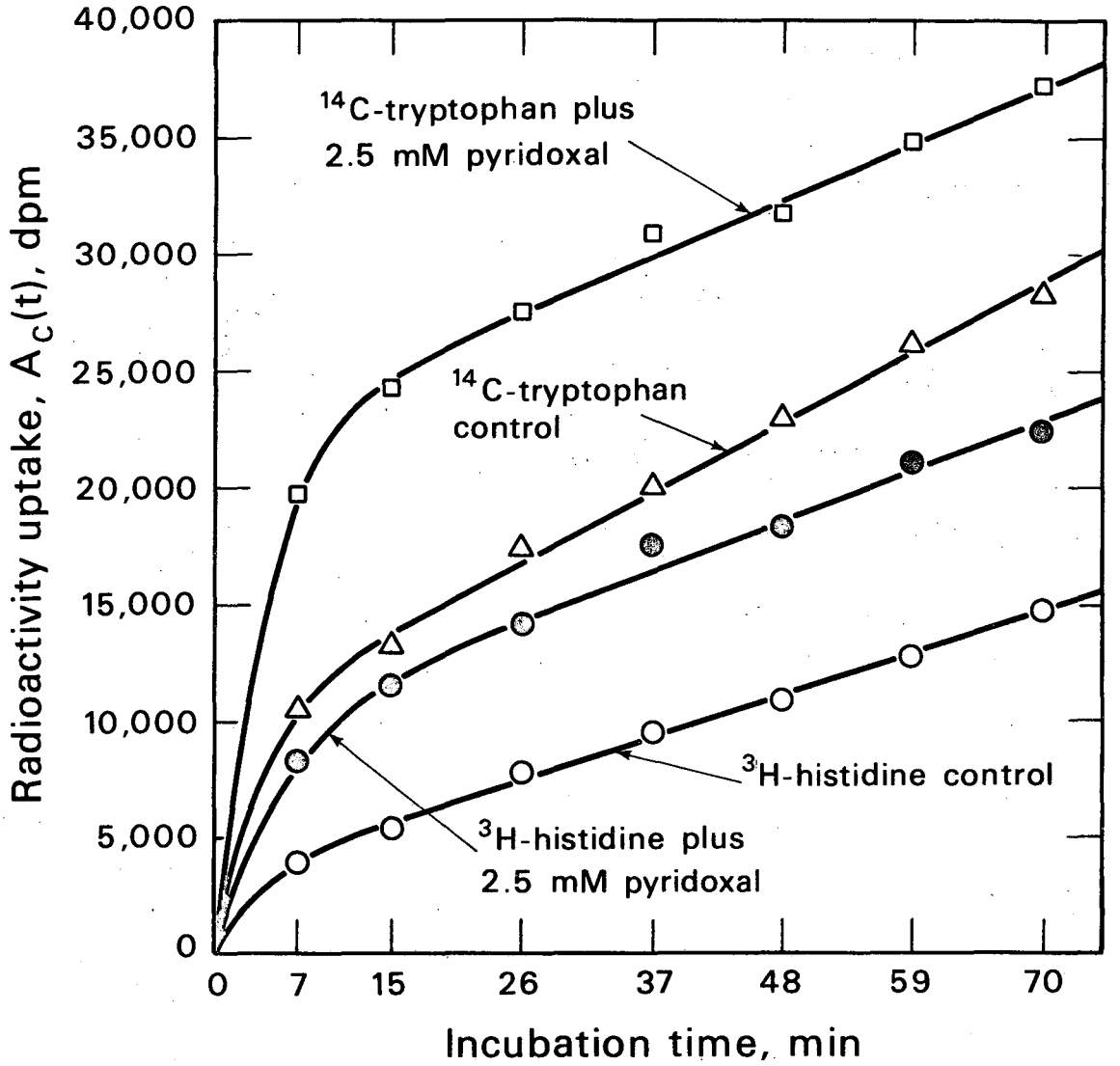

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Fig. 10

extent of hemolysis between control and pyridoxal in this experiment is shown in TABLE VII. Results of compartmental analysis (TABLE VIII) seemed to indicate the following: (i) For both histidine and tryptophan, pyridoxal consistently increased both influx and efflux, expanded the "intracellular pool size", and decreased the fractional metabolic flux. (ii) Hypertrophy of the intracellular pool in the presence of pyridoxal appeared to result in diminished fractional metabolic flux. Diminished fractional efflux and fractional turnover rate of the intracellular pool in two experiments seemed to be due to excessive hypertrophy of the intracellular pool. These findings, however, must be regarded as tentative because of the different extents to which erythrocytes were lysed during the washing.

The following observations were then made to evaluate the effect of pyridoxal on tryptophan uptake by erythrocytes from venous blood of a normal human subject. Cellular ^{14}C accumulation from tryptophan-methylene-^{14}C in the presence of 2.5 mM pyridoxal in the incubation medium was found to be slightly higher than corresponding control values (TABLE IX). The uptake appeared to be very rapid with a halftime of less than 2 min. These findings were felt to account at least partially for the findings presented in Fig. 10 and TABLE VIII.

2. <u>Effects of Pyridoxal on Osmotic Fragility of Human Erythrocytes</u>

The findings of apparent ability of pyridoxal to de-

TABLE VII

Effects of Pyridoxal on Hemolysis of Dog Bone Marrow Erythrocytes during Incubation in Basal Medium of Eagle (BME) and during Subsequent Washing in Ammonium Oxalate Solution.

Hemolysis during Optical density (OD) at 541 mu of	Incubation Supernatant Medium	Washing Ammonium oxalate wash
	OD	OD
Control (no pyridoxal in medium)	0.042	0.171
Pyridoxal (2.5 mM) in medium	0.020	0.029

Following incubation of 16 x 10^7 erythrocytes and 5.2 x 10^7 nucleated cells in the absence and presence of pyridoxal (2.5 mM) in 5 ml BME for 70 min with shaking (140 cycles/min), cells were packed by centrifugation and washed once in 5 ml 80 mM ammonium oxalate solution. Optical density of the 5 ml supernatant medium and 5 ml supernatant wash were then read against appropriate blanks.

TABLE VIII

Effects of Pyridoxal (2.5 mM) on the Kinetics of the Intracellular Pools of L-Histidine and L-Tryptophan in Dog Bone Marrow Cells Suspended in BME.

	Fractional turnover rate of intracellular pool (r)	Influx (I)	Fractional efflux (k_{21})	Fractional metabolic flux (k_{23})	Intracellular pool size (I/r)	Efflux ($k_{21}K/r$)
	%	%	%	%	%	%
Histidine						
Exp. 1	77	137	78	43	179	139
Exp. 2	158	227	178	70	143	254
Exp. 3	66	181	70	43	276	193
Tryptophan						
Exp. 1	100	146	103	28	146	150
Exp. 2	211	297	223	63	141	314
Exp. 3	84	179	88	38	214	188

The values listed are presented as % of the corresponding control values. For reasons given in the text, these findings were felt to be tentative only.

TABLE IX

^{14}C Accumulation in Human Erythrocytes Incubated with L-Tryptophan-Methylene-^{14}C at 0.024 mM (SA 21 dpm/uumole) in the Absence and Presence of Pyridoxal (2.5 mM) in Basal Medium of Eagle*

	Control	Pyridoxal
Radioactivity uptake	dpm	dpm
at 2 min	334	388
5 min	356	404
10 min	374	420
20 min	434	626

*There were initially 9.0 x 10^7 erythrocytes present in the cell suspension. Following incubation, cells were recovered on a membrane filter and assayed for radioactivity content. A fraction of the total cellular radioactivity was lost to the filtrate due to minor cell breakage during the filtration under suction.

crease hemolysis in the preceding results prompted the following experiments.

Erythrocytes were obtained from venous blood of a normal human subject, washed and suspended in physiological saline solution, and then immediately subjected to fragility test in a series of isotonic solutions at equal concentration of ammonium oxalate but at varying concentrations of pyridoxal as described under MATERIALS AND METHODS. All solutions were osmotically equivalent to 141-142 mM NaCl solutions. Results were given in TABLE X. The presence of ammonium oxalate (57 mM) in the solution resulted in 81-84% hemolysis after 5 min independent of the presence of pyridoxal (0-0.71 mM). This finding, when contrasted with the previous findings (TABLE VII), suggested the following: (i) Increased resistance to lysis in 80 mM ammonium oxalate in distilled water (hypotonic) was imparted to the erythrocytes (from dog bone marrow) during incubation in the presence of pyridoxal prior to exposure to the ammonium oxalate solution. (ii) Resistance to lysis of erythrocytes (from human blood) by 57 mM ammonium oxalate under isotonic conditions was not imparted by the mere presence of pyridoxal in the solution.

To evaluate whether the pyridoxal truly decreased osmotic fragility of erythrocytes, tests of osmotic fragility in hypotonic saline solutions were carried out on human erythrocytes which had been incubated for 30 min in the absence and presence of pyridoxal (2.5 mM). As shown in Fig. 11,

TABLE X

Lysis of Human Erythrocytes by Ammonium Oxalate (57 mM) in the Presence of Pyridoxal (0-0.71 mM) under Isotonic Conditions (Equivalent to 141-142 mM NaCl Solutions).*

Pyridoxal concentration	Optical density at 541 mμ	% hemolysis
mM		%
0.71	0.434	83
0.36	0.435	84
0.071	0.428	82
0.036	0.436	84
0.0071	0.432	83
0.0036	0.425	82
0	0.422	81

* The initial erythrocyte concentration of the mixture was 0.2% (v/v). After standing the mixture for 5 min at room temperature, optical density of the hemolysate was read and further converted to % hemolysis. Hemolysis in 0.24% (w/v) NaCl was taken to be 100% hemolysis. Results given were means of duplicates.

FIGURE 11

Osmotic fragility of human erythrocytes - Effect of prior incubation in the presence of pyridoxal. In each experiment, erythrocytes were isolated from venous blood of a healthy adult and incubated in Gey's BSS with and without pyridoxal (2.5 mM) in the medium at 37°C for 30 min. Following the incubation, cells were recovered by centrifugation and washed twice in Gey's BSS. The incubated and washed cells were then subjected to osmotic fragility test in buffered NaCl solutions by a standard method. Optical density of hemolysate was read at 541 mμ and the reading was converted to % hemolysis taking hemolysis in distilled water as 100% hemolysis. Results from 4 experiments in 3 subjects are shown together in this figure. Closed (control) and open (pyridoxal) circles represent individual readings.

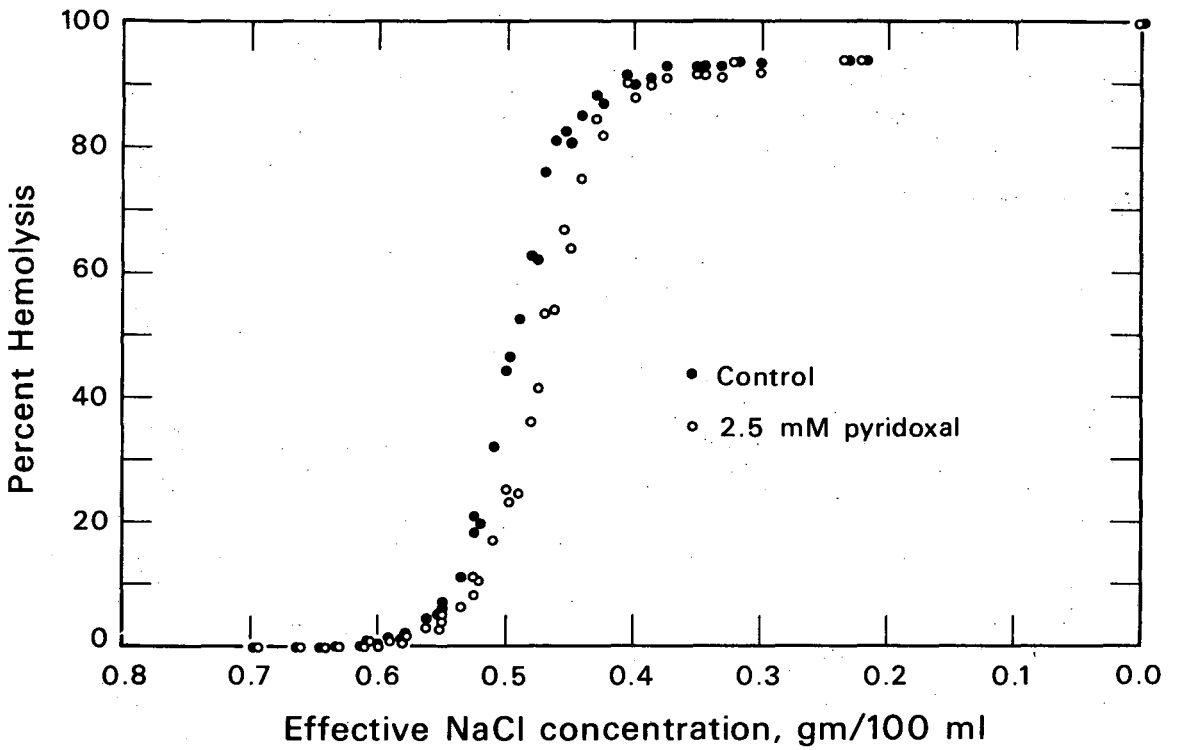

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Fig. 11

the presence of pyridoxal during the preceding incubation resulted in a slight shift of %-hemolysis curve toward low concentration side of control curve. The significance of this finding was discussed under DISCUSSION.

D. DISCUSSION

Ammonium oxalate solution has long been used to lyse erythrocytes in counting blood platelets (38). Erythrocyte membrane is probably only slightly permeable to ammonium ions but highly permeable to oxalate ions and uncharged ammonia. Ammonia forms in a solution of ammonium salt, enters the cell, and reforms ammonium ions and hydroxyl ions by hydrolysis within the cell. Anions, such as chloride ions, enter the cell in exchange for the hydroxyl ion. In this manner, it is believed (39, 40), erythrocytes are osmotically lysed in solutions of ammonium salts. A solution of ammonium salt with sufficiently low pH and hence little formation of ammonia in the solution may not have hemolytic effect. This, however, is unlikely the basis for the present finding of failure of ammonium-oxalate washing to lyse erythrocytes previously exposed to pyridoxal, because all solutions of ammonium oxalate (80 mM) containing 0-1 mM pyridoxal as prepared in this study (cf. MATERIALS AND METHODS) have pH in the 6.4-6.5. Another possible explanation for the finding is that pyridoxal forms Schiff's bases with many amines (41). If pyridoxal should form a Schiff's base with ammonia inside erythrocytes, virtual entry of ammonium salt into the cells might be effectively minimized.

This, again, is unlikely the case. Otherwise, in the experiment presented in TABLE X, the extracellular pyridoxal may be expected to minimize entry of ammonia into the cell, and thus there may be no cause for hemolysis to occur in the first place, contrary to the results (TABLE X).

In contrast to the remarkable resistance of erythrocytes preloaded with pyridoxal to hemolytic effect of ammonium oxalate, similarly preloaded erythrocytes show only slight increase in resistance to osmotic lysis in hypotonic saline solutions (Fig. 11). Accordingly, the former finding appears to represent merely an apparent decrease in osmotic fragility of erythrocytes. Even the latter finding may not be associated with true alteration in osmotic fragility of erythrocyte membranes. As stated under MATERIALS AND METHODS, the present preparation of incubation mixture containing pyridoxal involves neutralization of pyridoxal hydrochloride with NaOH and subsequent dilution in medium used to prepare the control incubation mixture. Thus, the extracellular fluid containing 2.5 mM pyridoxal may have an osmolar concentration higher than the control value by an equivalent of as much as 5 mM NaCl. It is possible, therefore, that the observed difference between the two hemolysis curves in Fig. 11 is related to a slight dehydration of erythrocytes previously incubated in medium containing neutralized pyridoxal hydrochloride. Somewhat against this view stand the following considerations: (i) Before the incubated erythrocytes are subjected to osmotic fragility

test, they are washed twice in medium used to prepare the control incubation mixture. It is likely that minor differences in cell hydration would be eliminated during the washing. (ii) During the incubation, extracellular pyridoxal can form complexes with alkali metal ions, complexes with amino acids, and complexes with both (42). If most of the pyridoxal should exist as the three-member complexes, osmolar concentration of the extracellular fluid containing pyridoxal would actually be lower than control values. This seems impossible, because there is not enough amino acid in the medium (Gey's BSS) to permit formation of 2.5 mM hypothetical three-member complexes. However, there are certainly more than enough sodium, potassium, and calcium ions to permit formation of 2.5 mM two-member complexes. (iii) Pyridoxal enters the cell and may even concentratively accumulate within the cell (35). Under the circumstances, the direction of alteration, if any, in the balance of osmotic concentration across the erythrocyte membrane is by no means clear without measurement of changes in cell volume. The most substantial argument in support of a slight but real change in osmotic fragility of pyridoxal-loaded erythrocytes independent of cell volume change prior to fragility test is this --- In all likelihood, water movement across the erythrocyte membrane is sufficiently fast that minor differences in erythrocyte volume at the beginning of a 45 min exposure of the erythrocytes to 400 volumes of fragility test solutions would make no detectable difference in

the outcome of the test. At any rate, prior to the 45 min fragility test, the erythrocytes are incubated for only 30 min. Regardless of how fast or how slow water moves across the cell membrane, 45 min is longer than 30 min.

The pyridoxal concentration of 2.5 mM has been chosen for this study since it was felt to correspond in a way to the administration of 100 mg pyridoxine hydrochloride to a 250 gm rat in Ngo's *in vivo* study previously referred to under INTRODUCTION. It is tempting to speculate that the diminished $^{14}CO_2$ production from tryptophan-1-^{14}C in his observation is related to ^{14}C dilution in an intracellular tryptophan pool already expanded by prior administration of pyridoxine hydrochloride. Several questions remain to be satisfied by such speculation. It is not known how fast pyridoxine is converted to active pyridoxal or pyridoxal phosphate *in vivo*. Only the latter two aldehyde forms of vitamin B_6 stimulate amino acid uptake in Ehrlich ascites tumor cells (35). Secondly, contrary to Ngo's *in vivo* findings, selective effect on tryptophan is not the case in all *in vitro* studies known to this author. Thirdly, under *in vivo* conditions, the amount of amino acids present extracellularly is very small compared to that present intracellularly. It is difficult to understand how an intracellular amino acid pool can expand substantially in a matter of 30 min in an animal. On the other hand, Ngo's finding may be taken as suggesting an interesting possibility, namely, that *in vivo* there exists an organ (e.g., the liver)

or tissue only which significantly converts the carboxyl carbon of tryptophan to CO_2, and further, as a result of massive pyridoxine administration, only whose intracellular tryptophan pool is substantially enlarged.

The majority of clinical cases of pyridoxine-responsive anemia are associated with hypochromia of erythrocytes, which, being "poorly filled", tend to have increased resistance to hemolysis in hypotonic saline solutions. Such consideration indicates that the yet unknown reason for the efficacy of massive pyridoxine therapy in this clinical syndrome is to be sought not in connection with osmotic fragility of erythrocytes but elsewhere.

E. SUMMARY

Intracellular accumulation of radioactivity from L-histidine-imidazole-2,5-^{3}H and L-tryptophan-methylene-^{14}C by dog bone marrow nucleated cells incubated in Basal Medium of Eagle was measured and analyzed according to a three-compartment model. For both amino acids, pyridoxal administered at 2.5 mM concentration to the culture medium appeared to stimulate both influx and efflux, expand the intracellular pool, and diminish the fractional metabolic flux. These findings were considered presumptive, because contaminating erythrocytes exposed to pyridoxal during the incubation, in contrast to those from control incubation, were not adequately lysed during washing in ammonium oxalate solution subsequent to the incubation. Prior exposure of erythrocytes to pyridoxal but not the presence of pyridoxal in

ammonium oxalate solution appeared to have caused the diminished hemolysis in the ammonium oxalate solution. Normal human erythrocytes similarly exposed to pyridoxal were found to have slightly decreased osmotic fragility in hypotonic saline solutions apparently independent of any cell volume change prior to the fragility.

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