the two tissues. High pyruvate concentrations inhibit LDH activity from either source to the same extent. The degree of inhibition by urea is always significantly less ($p<.005$) at the high (1×10^{-2} M) than at the low (5×10^{-4} M) pyruvate level. Oxalate inhibits inguinal adipose tissue LDH less at the high than at the low pyruvate level, while showing a reverse effect on the enzyme from pericardial tissue. Michaelis-Menten constants (K_M) for pyruvate were calculated from activities both in the absence and in the presence of urea and oxalate. K_M values were significantly decreased in the presence of 2.0 M urea. All levels of oxalate studied increased the K_M of pericardial adipose tissue LDH, but did not affect that of the inguinal adipose tissue enzyme. Incubation at 59°C for 10 minutes inactivates both enzymes to about the same extent, leading to a loss of 44 and 47% of their activity, respectively. Electrophoretic separation revealed 5 isozymes with a higher proportion of fast moving subunits in pericardial adipose tissue LDH.

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Transport of Alanine, Arginine and Glutamic Acid in the Rabbit Erythrocyte and Reticulocyte.* (31813)

HAROLD C. ROHRS† AND J. W. ARCHDEACON
(Introduced by L. D. Carlson)
Department of Physiology and Biophysics, University of Kentucky College of Medicine, Lexington

Early studies of Christensen *et al* reveal that the nucleated duck red blood cell and the human erythrocyte are able to maintain higher intracellular concentrations of apparently free amino acids than concentrations found in plasma(1). Riggs *et al* found the rabbit reticulocyte has an intracellular glycine content 3 to 5 times greater than the plasma (2). In contrast, the rabbit erythrocyte is un-

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† Present address: Dept. of Zoology, Drew University, Madison, N. J.

able to concentrate glycine. Recent studies by Winter and Christensen show that transport differences between neutral amino acids in the rabbit erythrocyte and reticulocyte are the result of a cessation or loss of specific processes in cell transition(3). Yunis and Arimura studied transport features of several amino acids in the rat reticulocyte and mature red blood cell(4). These workers found the mature cell has little or no capacity to concentrate amino acids against gradients.

In the present study, the distribution ratio, *i.e.*, the concentration of labeled intracellular amino acid divided by the concentration of labeled extracellular amino acid, was determined for an acidic (glutamic acid), a dibasic (arginine) and a small neutral (alanine) amino acid in the erythrocyte and reticulocyte during 30 minutes incubation at 37°C. Blood cells were obtained from normal adult rabbits and rabbits injected with phenylhydrazine. Variance in uptake behavior was observed in the two kinds of cells for each amino acid. Also cells showed differences in uptake characteristics when they were treated with iodoacetic acid. Some attention in directed toward a quantitative consideration of uptake kinetics in the *Discussion*.

Materials and methods. Uniformly labeled L-alanine-C^{14},‡ L-arginine-C^{14},‡ and L-glutamic acid-C^{14}§ and the corresponding non-labeled forms were used. Adult New Zealand rabbits of both sexes were the experimental animals. Increased population of reticulocytes in the blood was induced with 2.5% aqueous phenylhydrazine injected intraperitoneally (1 ml) for 4 consecutive days. Blood (15 ml) was drawn by cardiac puncture on the seventh day. Microscopic examination of blood smears was made with new methylene blue(5). Hematocrits of phenylhydrazine injected rabbits ranged from 15-20 in contrast to untreated animals with 50-60.

The sample of blood, with 1 ml of sodium heparin solution (1,000 U.S.P. Units/ml) added, was centrifuged 5 minutes at 750 × *g*. Plasma and buffy coast were aspirated. The cells were washed 3× with approximately 10 ml of 0.15 M NaCl solution (2× cell volume added each washing). Supernatants were discarded. 1.0 ml of packed cells was introduced into a flask containing 99.0 ml of 0.15 M NaCl solution. They were mixed thoroughly shaking by hand. 1.0 ml of the cell suspension was introduced into each of 36 test tubes and these were centrifuged 1 minute. Supernatant fluid was removed and replaced with isotonic fluid containing 125 mM sodium chloride, 5 mM potassium chloride, 1 mM magnesium chloride, 8.2 mM glucose and 30 mM tris (2-amino-2-(hydroxymethyl)-1,3-propanediol) chloride, pH 7.4. The selected non-labeled amino acid was added to the solution in 0.1 mM concentration. A tracer amount of this amino acid, C^{14} labeled, was added until the radioactive count was calculated at 50,000 counts/minute/ml. Separate groups of 4 tubes were incubated 0, 0.5, 1, 1.5, 2, 5, 10, 20 and 30 minutes.

The tubes and contents were agitated (60-70 strokes/minute) in a Warburg bath at 37°C. The gas medium was air. After incubating, each tube and its contents were centrifuged 1 minute and supernatant was aspirated. The cells were resuspended in 2.0 ml of a solution of the same composition as the original bathing fluid minus the radioactive amino acid. This preparation was recentrifuged and supernatant fluid aspirated. Tenenhouse and Quastel, using a similar method of washing Ehrlich ascites cells, observed that one washing effectively removed trapped extracellular radioactive amino acid without affecting intracellular concentration(6). Finally the packed cells were hemolyzed by adding 2.0 ml of water. This preparation was centrifuged 1 minute and 1.0 ml of the lysate was removed and allowed to flow on an aluminum planchet. After drying in room air, the preparation was assayed for radioactivity with a Packard gas-flow detector and a Nuclear-Chicago 181 decade scaler.

Treatment with iodoacetic acid (IAA). The effect of 10^{-3} M IAA was observed on uptakes of the three C^{14} labeled amino acids by the erythrocyte and reticulocyte. Thirty-six tubes each containing 1 ml of the cell suspension were prepared as described. After centrifugation and removal of the supernatant

‡ Schwarz BioResearch, Inc.

§ Volk Radiochemical Co.

fluid cells were resuspended in 1 ml of a solution containing 0.15 M NaCl, 8.2 mM glucose and IAA. Control preparations did not have the metabolic inhibitor. The cells were incubated at 37°C for 1 hour. This preincubation treatment with IAA was carried out attempting to exhaust cellular synthetic sources of metabolic energy. Subsequently the cells in each tube were resuspended in 1 ml of the original bathing medium containing salts, glucose, tris chloride, selected amino acid, labeled and unlabeled, in concentrations presented previously, and 10^{-3} M IAA (excepting controls). The cells were incubated again and assayed for radioactivity in the manner outlined in the original treatment.

Chromatographic analysis. In several experiments larger volumes of cells were used (10×) and lysates were chromatographed on Whatman #1 filter paper in an ascending system with butanol-acetic acid-water. Location of radioactivity was made by counting 1 cm serial strips in the gas-flow counter. Results show that each labeled amino acid was freely extractable and apparently not bound to protein. In addition, cellular protein residue showed no radioactivity.

Amino acid distribution ratio. The amino acid distribution ratio (D.R.) was calculated using the formula:

$$\text{D.R.} = \frac{S_c}{A_o\,(V_T - V_{inulin})}$$

in which A_o is extracellular amino acid activity in counts/minute/ml; S_c is radioactivity of the lysate in counts/minute; V_T is packed cell volume; and V_{inulin} is trapped extracellular or inulin space. The latter value was determined using uniformly labeled C^{14} inulin.‖ It was 30.0% (± S.E. of 1.6) of the packed cell volume for erythrocytes and 30.1% (± S.E. of 1.1) for reticulocytes. Blood cells from rabbits not injected with phenylhydrazine were considered as entirely erythrocytes. Erythroid cells from injected animals consisted of both reticulocytes, 59.7% (± S.E. of 2.9) and erythrocytes. The distribution ratios for reticulocytes were computed as those of 100% reticulocytes after correction for ratios of the admixing erythrocytes. The assumption was made that the erythrocyte of the phenylhydrazine treated animal had the same ratio for the selected amino acid as the erythrocyte of the normal animal.

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Results. Distribution ratios. The uptakes of C^{14}-labeled alanine, arginine and glutamic acid are plotted as distribution ratios during 30 minutes. These values are shown for the erythrocyte in Fig 1 and the reticulocyte in Fig. 2. Even with rapid manipulation approximately 1 minute was required to separate cells from the fluid medium and institute the washing procedure. Consequently the values, as shown are greater than 0 at graphed 0 time.

Marked differences appeared in uptakes of alanine and arginine in the two erythroid forms. Arginine concentration increased early in the erythrocyte, whereas, after an initial brisk accumulation, it decreased in the reticulocyte. Alanine uptake in the erythrocyte was not as great as in the reticulocyte. This amino acid accumulated rapidly in the latter cell and had highest concentration of the amino acids. The distribution ratios for glutamic acid in the erythrocyte did not indicate accumulation against a gradient during 30 minutes. However, the ratio of this amino acid in the reticulocyte was slightly more than 1 after 20 minutes.

Effect of IAA. The distribution ratios of the amino acids in the rabbit erythrocyte and reticulocyte, when they were treated with 10^{-3} M IAA as described in *Methods,* are shown in Figs. 3 and 4 respectively. The control data are indicated by solid lines. First, the arginine values in the erythrocyte were reduced solely by the 1-hour preincubation at 37°C (without IAA). This is apparent when the graphed data in Fig. 3 are compared with those for arginine in Fig. 1. Also, the initial burst of arginine uptake in the reticulocyte, as shown in Fig. 2, disappeared after similar treatment. Alanine and glutamic acid uptakes were not modified appreciably without IAA in either the erythrocyte or the reticulocyte. However, as the graphs reveal, treatment with IAA caused

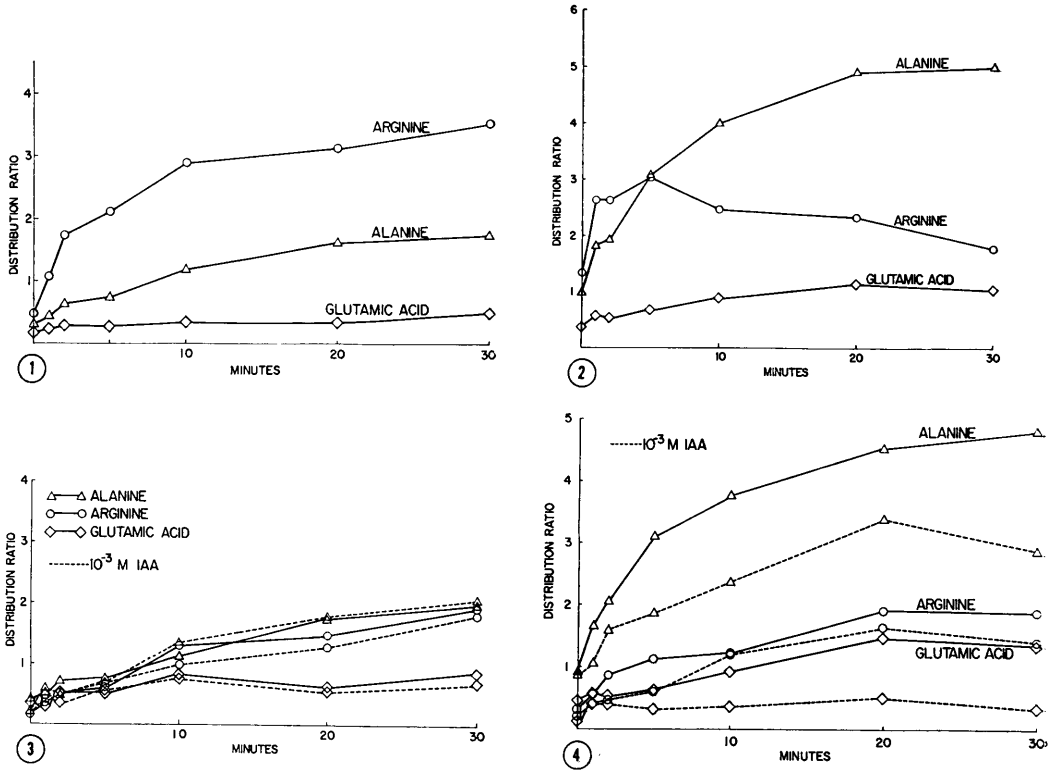

FIG. 1. Distribution ratios of C^{14}—labeled amino acids in erythrocytes incubated at 37°C.
FIG. 2. Distribution ratios of C^{14}—labeled amino acids in reticulocytes incubated at 37°C.
FIG. 3. Distribution ratios of C^{14}—labeled amino acids in erythrocytes incubated with IAA at 37°C.
FIG. 4. Distribution ratios of C^{14}—labeled amino acids in reticulocytes incubated with IAA at 37°C.

marked inhibition of alanine and glutamic acid uptakes in the reticulocyte, probably due to the inhibitor blocking anaerobic metabolic processes. The erythrocyte was not affected.

Discussion. It is apparent in these studies that the rabbit erythrocyte and reticulocyte displayed differences in uptake kinetics for the 3 amino acids. The net direction of movement of arginine was reversed early in the two cells, accumulating in the erythrocyte and decreasing in the reticulocyte. Obviously a transport scheme for arginine was not operating efficiently in the latter cell. Alanine and glutamic acid accumulated in greater quantities in the reticulocyte. Winter and Christensen showed that transport differences between neutral amino acids in the rabbit erythrocyte and reticulocyte are due to losses of specific mechanisms, one of which has a high affinity for alanine(3). Similarly, perhaps decay of a mechanism for glutamic acid transport is responsible for the failure of the erythrocyte to accumulate this amino acid. The reason for the difference in transport behavior of arginine in the two cells in not apparent.

The ability of the erythrocyte treated with IAA to concentrate alanine and arginine may be associated with exchange of intracellular K. Konsek and Bishop showed that when human erythrocytes are exposed to IAA (6×10^{-4} M) high energy phosphate stores of the cells are depleted before loss of high intracellular K(10). Riggs *et al* suggested that amino acid uptake by Ehrlich ascites cells is driven by the energy inherent in the asymmetry of the transmembrane K ion distribution(11). A residual Na ion gradient may be implicated since Vidaver demonstrated that pigeon red cells can

achieve a glycine concentration only if a Na gradient exists across the cell membrane(12, 13). The observation that arginine uptake by the erythrocyte and the reticulocyte was reduced solely by a 1-hour preincubation of the cells may indicate that some source or sources of energy for supporting accumulation of this amino acid, even though glucose is present in the incubating fluid, becomes limited or exhausted by warming at 37°C.

The reason for the change in shape of the arginine uptake curve for the reticulocyte is not apparent at present. Perhaps the early elevated uptake is related to a high transmembrane cation gradient. An alternate hypothesis is that exchange diffusion took place between intracellular amino acids and labeled extracellular arginine.

Kinetic analysis. Rosenberg *et al*(7) observed that the amount of amino acid accumulated by a segment of everted intestine at any given time is related to the maximal amino acid uptake and is given by the equation:

$$u = u_\infty(1-e^{-kt}) \qquad (1)$$

in which u is the uptake at time t, u_∞ is the maximal uptake, k is the rate constant, and t is the incubation time. Robertson(8) has shown that the specific activity x_B of a compound in one compartment of a two-compartment system is related to the specific activity x_A of the compound in the second compartment by the equation:

$$x_B = x_A(1-e^{-kt}) \qquad (2)$$

This equation is valid only if the labeled compound is instantaneously introduced into compartment A and if the volume and amount of compound in compartment A is much larger than in compartment B.

In the system described in equation (1), the amount of amino acid inside the intestinal segment (the compartment in which amino acid uptake is being observed) is zero when $t = 0$. Equation (2) requires only that there be no labeled compound at $t = 0$ in the observed compartment. In the present study, measurements were made of the amount of labeled amino acid accumulated by cells which probably contained unlabeled amino acid prior to incubation. Extracellular amino acid was in the physiological range in rabbit serum(9). Changes in intracellular radioactivity are expressed as changes in intracellular amino acid concentrations and ultimately distribution ratios. This method of determining amino acid uptake seems justified since the equations of Rosenberg *et al* and Robertson are identical in form.

Movement of an amino acid between two compartments may be expressed by the equation:

$$C_i = \left(\frac{A + BC_o}{B}\right)(1-e^{-Bt}) \qquad (3)$$

in which A is the rate of amino acid uptake into the cell divided by the intracellular volume, B is the amino acid efflux coefficient divided by the intracellular volume. C_i is the intracellular amino acid concentration, C_o is the extracellular amino acid concentration and t is incubation time. This equation is derived assuming amino acid concentration in the cell involves (1) a constant rate of amino acid influx (occurring by means of a pump-like mechanism from the medium into the cell), and (2) an efflux of accumulated amino acid from the cell into the medium by diffusion. Derivation of equation (3) is presented in the *Appendix*.

Semilogarithmic plots of the slopes of alanine uptake curves in both erythrocytes (Fig. 1) and reticulocyte (Fig. 2) against time yield straight lines. This strongly indicates that alanine movements occurred between two compartments and is expressed by equation (3). Also, slopes of uptake data similarly plotted for arginine accumulation in the erythrocyte yield a straight line. Due to early arginine loss from the reticulocyte slopes of this curve plotted semilogarithmically do not form a straight line. However, arginine slopes for IAA treated reticulocytes (Fig. 4), when plotted semilogarithmically with respect to time, form a straight line (Fig. 5) again indicating simple, two-compartment exchange.

Summary. Characteristics of uptake of alanine, arginine and glutamic acid have been studied in the rabbit erythrocyte and reticulocyte. Evidence is presented that the reticulocyte posseses a mechanism or mechanisms for transport of alanine and glutamic acid that are lacking in the erythrocyte. The

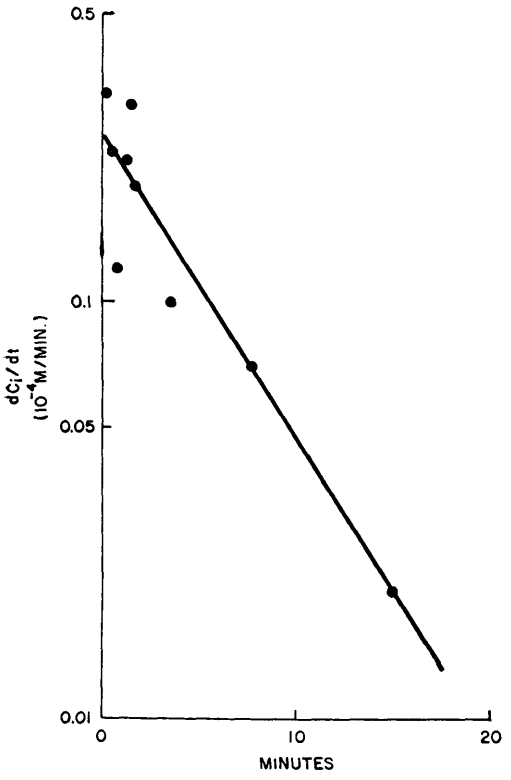

FIG. 5. Semilog plot of slopes of arginine uptake in IAA incubated reticulocytes.

sources of supporting energy are blocked by exposure to IAA. Arginine accumulates to a greater extent in the erythrocyte than the reticulocyte. A quantitative analysis of amino acid uptake is presented.

Appendix. Derivation of equation.¶ C_i and C_o are the intracellular and extracellular amino acid concentrations; K is the efflux coefficient of the amino acid; $\dot{q}_c$ is the net rate of intracellular amino acid accumulation; $\dot{q}_p$ is the pump rate; V_i is the intracellular volume and t is time. C_o is considered to remain relatively constant throughout incubation.

$$\dot{q}_c = \dot{q}_p + K(C_o - C_i)$$

$$\Delta C_i = \dot{q}_c \Delta t / V_i$$

$$dC_i/dt = \dot{q}_c/V_i = \dot{q}_p/V_i + (K/V_i)(C_o - C_i)$$

$$\int_0^{C_i} dC_i / [(-KC_i/V_i) + (KC_o/V_i) + (\dot{q}_p/V_i)] = \int_0^t dt$$

$$\ln \frac{(-KC_i/V_i) + (KC_o/V_i) + (\dot{q}_p/V_i)}{(KC_o/V_i) + (\dot{q}_p/V_i)} = -\frac{K}{V_i} t$$

Let $A = \dot{q}_p/V_i$; and $B = K/V_i$

$$C_i = \frac{-(A + BC_o)\, e^{-Bt} + A + BC_o}{B}$$

$$C_i = \left(\frac{A + BC_o}{B} \right) (1 - e^{-Bt})$$

¶ Dr. Joseph Engelberg, Dept. of Physiology and Biophysics, Univ. of Kentucky, generously aided in the development of this equation.

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