The Uptake of Tyrosine by Isolated Rat Diaphragm

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It is now recognized that cells in vivo are able to maintain intracellular concentrations of free amino acids higher than those of the circulating plasma (1). Extensive studies have been conducted on amino acid transport in the kidney (2) and the intestine (3). Uptake by brain in vivo has also been studied (4-6). However, the exact mechanisms of amino acid entry into the cell and for maintenance of elevated intracellular concentrations remain obscure.

Mechanistic studies are more feasible in isolated systems, particularly those allowing observation of groups of like cells in uniform suspension. Both red blood cells (7) and ascites tumor suspensions (8, 9) have served in this regard, but the specialized nature of these systems makes widespread generalization questionable. The tissue slice has served as a transport model (10), as has the excised diaphragm (11, 12). Reports have appeared recently concerning the uptake of the model amino acid α-aminoisobutyric acid into cells of isolated, intact diaphragm (13, 14).

Inasmuch as knowledge of the mode of transport into mammalian cells also requires precise studies on the uptake of naturally occurring metabolites, the present studies concerning the uptake of the amino acid tyrosine by rat diaphragm in vitro were undertaken. They show that a process of diffusion is responsible for tyrosine entrance into this tissue. Several lines of evidence indicate that, within the diaphragm, an amount of tyrosine comparable to the fasting endogenous concentration is not in rapid diffusion equilibrium with the external medium.

EXPERIMENTAL PROCEDURE

Male Sprague-Dawley rats weighing between 130 and 150 g were used throughout. The animals were fasted for 18 hours before each experiment. They were decapitated and their diaphragms removed by the "intact" diaphragm technic of Kipnis and Cori (15), with retention intact of the insertions of the diaphragm to a circular section of body wall. The intact diaphragms were rinsed in buffer at room temperature for a few seconds and drained before incubation. "Zero-time" diaphragms were rinsed in buffer at room temperature, blotted on filter paper, and weighed on a torsion balance. They were homogenized with 15 ml of 0.2 N HCl per g of tissue in a VirTis "45" homogenizer for 1 minute. The homogenates were then deproteinized with 4 ml of 30% trichloroacetic acid per g of tissue. Clear extracts were obtained by preliminary filtration through glass wool followed by centrifugation. The incubation buffers were deproteinized with 1 part of 30% trichloroacetic acid per 4 parts of buffer.

Tyrosine was estimated by the spectrophotofluorometric method of Waalkes and Udenfriend (16) and tryptophan by the method of Duggan and Udenfriend (17). In the radioisotope measurements, samples were counted in a Packard TriCarb liquid scintillation counter with the anthracene-aqueous phase counting technique of Steinberg (18). Inulin was determined by the Roe method (19). L-Tyrosine was purchased from Nutritional Biochemical Corporation. d-Tyrosine was kindly provided by Geigy Chemical Corporation. Both isomers were shown to be pure by chromatographic procedures; their optical purity was verified by treatment with snake venom L-amino acid oxidase. Uniformly labeled L-tyrosine was obtained from Nuclear Instrument Company and its purity confirmed by paper chromatography.

Intracellular concentration \(c_i\) was calculated with the equation:

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c_i = \frac{c_t - c_b \cdot E}{1 - E}
\]

in which \(c_t\) is the concentration per ml of tissue water, \(c_b\) is the concentration in the incubation medium, and \(E\) is the ratio of inulin space to total tissue water (15). "Intracellular uptake" refers to the increase in the intracellular concentration above the endogenous value.

RESULTS

Characteristics of Diaphragm Preparation—Total water and inulin space measurements were performed in separate experiments on diaphragms incubated for 90 minutes in saline-bicarbonate buffer containing 1 mm L-tyrosine. These measurements were averaged, the \(E\) value calculated, and this \(E\) value used in...
the calculation of the intracellular concentrations in the succeeding experiments. The total water content remained constant at 78.4 ± 0.5 (s.e.) ml per 100 g of wet tissue during the incubation. The inulin space reached approximately 9% of the wet weight of the tissue by 30 to 50 minutes but increased slowly (up to 13% of the wet weight) over the rest of the incubation period. An average figure for the inulin space between 70 and 90 minutes was used in the calculation giving an inulin space of 11.7 ± 1.7 (s.e.) ml per 100 g of wet tissue. These spaces yield an E value of 0.15.

The extent of tyrosine metabolism by the diaphragm in these experiments was investigated with radioactive \( n \)-tyrosine. After a 90-minute incubation, a recovery of 99% of the activity of a tracer amount (<1 \( \mu \)g per ml) of tyrosine was obtained in the protein-free tissue extract plus the buffer medium. These extracts were found by paper chromatography to have only one radioactive component, with an \( R_F \) value identical to that of authentic tyrosine.

The diaphragm, as removed from the animal, contained 23 \( \mu \)g of tyrosine per ml of intracellular water and maintained nearly this concentration during 90-minute incubations in tyrosine-free buffer. However, when the intact diaphragm was incubated in tyrosine-free buffer, there was an increase in the tyrosine content of the buffer, presumably due to proteolytic activity. In other experiments, hemispheres incubated alone released a 90-minute incubation, a recovery of 96% of the activity of a comparable intracellular concentrations of tyrosine, intact diaphragms were incubated in 1.25 mM \( n \)- or 1.0 mM L-tyrosine for 60 minutes and then placed in tyrosine-free buffer (Fig. 2). The diaphragms released \( n \)-tyrosine about 30% more slowly than they did L-tyrosine. The difference between the isomers, however, was not as great as that seen in the uptake studies. The experiments with L-tyrosine showed that the diaphragm did not release its complete content of tyrosine, but retained approximately 30 \( \mu \)g per ml of intracellular water even though periodic renewal of the medium kept the external concentration below 1 \( \mu \)g per ml. The rates of release and the final equilibria were approximately equal to that of the buffer.

When diaphragm was exposed to a similar concentration of \( n \)-tyrosine, the intracellular rate of uptake was considerably slower (Fig. 1). The uptake of \( n \)-tyrosine after 50 minutes was approximately one-half as great as the uptake of L-tyrosine, although equilibrium seemed to have been reached in the same time. In these studies, the volume of \( n \)-tyrosine distribution was constant at 50 to 60% of the intracellular water.

**Release of Tyrosine from Diaphragm**—In order to obtain comparable intracellular concentrations of tyrosine, intact diaphragms were incubated in 1.25 mM \( n \)- or 1 mM L-tyrosine for 60 minutes and then placed in tyrosine-free buffer (Fig. 2). The diaphragms released \( n \)-tyrosine about 30% more slowly than they did L-tyrosine. The difference between the isomers, however, was not as great as that seen in the uptake studies. The experiments with L-tyrosine showed that the diaphragm did not release its complete content of tyrosine, but retained approximately 30 \( \mu \)g per ml of intracellular water even though periodic renewal of the medium kept the external concentration below 1 \( \mu \)g per ml. The rates of release and the final equilibria were essentially the same when L-tyrosine was accumulated to a concentration of 70 to 80 \( \mu \)g per g by the diaphragm in vivo and released in vitro.\(^1\) Other experiments have indicated that a fresh diaphragm preparation, incubated merely in tyrosine-free buffer at 37\(^\circ\), will maintain nearly its original tyrosine content (approximately 28 \( \mu \)g per ml) for 90 minutes or more. It appears, then, that the tissue is able to hold an internal tyrosine concentration, approximately equal to the fasting endogenous concentration, in a form which is not in rapid diffusion equilibrium with the medium. Subsequent experiments confirm this conclusion.

**Uptake of Tyrosine as Function of External Concentration**—The initial uptake of L-tyrosine from buffers containing increasing concentrations of tyrosine (Fig. 3) was linear with respect to concentration up to the limit of tyrosine solubility. Thus no evidence of a saturable mechanism was obtained. Reciprocal plots (Lineweaver-Burk) of these data extrapolate to the origin, indicating either an extremely large or an infinite \( V_{\text{max}} \). With external concentrations below 50 \( \mu \)g of tyrosine per ml, the results became unreliable because uptake by the tissue was quite low and proteolytic release of tyrosine raised the external concentration.

**Volume of Tyrosine Distribution** The volume of distribution at equilibrium after exposure to various concentrations of L-tyrosine (Fig. 4) was constant at about 75 to 80% of the intracellular water if the endogenous tyrosine was subtracted in each calculation of the intracellular concentration (lower curve). If this endogenous amount was not subtracted (upper curve) the

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\(^1\) G. Guroff, and S. Udenfriend, unpublished data.
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Fig. 3. Intracellular uptake of L-tyrosine by isolated rat diaphragm as a function of external tyrosine concentration. Diaphragms were incubated for 10 minutes in 30 ml of saline-bicarbonate buffer at 37°C under 95% O₂ and 5% CO₂. Each point represents the average of three diaphragms.

Fig. 4. Distribution of L-tyrosine in isolated rat diaphragm as a function of external tyrosine concentration. ▲-▲, total tyrosine distribution; ●-●, distribution of accumulated tyrosine (endogenous tyrosine subtracted from the total intracellular concentration). Diaphragms were incubated for 60 minutes in 30 ml of saline-bicarbonate buffer at 37°C under 95% O₂ and 5% CO₂. Each point represents the average of three diaphragms.

When the diaphragms were exposed to low concentrations of L-tyrosine-C¹⁴ (<1 μg per ml) in the external medium (Fig. 5) the intracellular uptake of radioactive tyrosine (curve) was the same with respect to the radioactivity of the buffer as was the intracellular uptake of 1 mM L-tyrosine (triangles) in comparable experiments. Not only did trace levels of tyrosine reach equilibrium at the same time but they also yielded the same volumes of distribution as were obtained with 1 mM L-tyrosine (excluding endogenous).

These experiments indicate, then, that external tyrosine, over a wide range of concentrations, will partition between tissue intracellular water and extracellular buffer in a ratio of about 0.8. Before this relationship can be observed, however, the fasting endogenous amount must be subtracted from the final total tyrosine of the tissue.

Nonequilibration of Endogenous Tyrosine with External Tyrosine—Although equilibrium between external and internal radioactivity was achieved after 50 minutes of incubation, the specific activity of the external tyrosine even after 90 minutes was still more than 8 times greater than that of the tissue (Table I). The difference would have been even greater except for proteolytic release of tyrosine which continually lowered the external specific activity. Thus, endogenous tyrosine was not in rapid equilibrium with added radioactive tyrosine.

Effect of Temperature—A study of the initial uptake from 1 mM solutions of L-tyrosine at various temperatures (Fig. 6) indicated that the process has a Q₁₀ of 1.4 between 27 and 37°C. The data appear to indicate a disproportionate enlargement of the Q₁₀ at low temperatures (15) but the extremely small uptake at 0°C makes calculation of a temperature coefficient in that range unreliable. Incubation at 50°C led to slower uptake than into corresponding diaphragms at 40°C, perhaps because of some nonspecific change in the cell membrane at this elevated temperature.

<table>
<thead>
<tr>
<th>Time</th>
<th>Intracellular uptake (c.p.m./ml)</th>
<th>Specific activity (c.p.m./μg tyrosine)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>intracellular (c.p.m./ml)</td>
<td>medium concentration (c.p.m./μg tyrosine)</td>
</tr>
<tr>
<td>min</td>
<td></td>
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<tr>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0.35</td>
<td>0.012</td>
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<tr>
<td>30</td>
<td>0.66</td>
<td>0.062</td>
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<tr>
<td>50</td>
<td>0.76</td>
<td>0.067</td>
</tr>
<tr>
<td>70</td>
<td>0.72</td>
<td>0.117</td>
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<tr>
<td>90</td>
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In this regard, when boiled diaphragms were incubated under these conditions the uptake was even slower but still significant.

**Effect of pH, Ionic Composition, Exogeneous Substrate**—Although the pH of the modified phosphate buffer was varied from 5.8 to 8.3, no pH optimum for initial tyrosine uptake could be shown. It was observed that media ranging in pH from 7.0 to 8.0 generally gave slightly lower values than those between 6.0 and 7.0. There was, however, considerable overlap in these experiments and the significance of these slight differences is doubtful.

Initial studies with complete Krebs-Ringer bicarbonate buffer and comparative experiments with the simple phosphate-saline medium indicated that the general characteristics of the uptake were the same regardless of the ionic composition employed. Although these experiments do not give a complete picture, they do rule out the gross participation of PO₄³⁻, Ca²⁺, Mg²⁺, and K⁺ ions.

The uptake of L-tyrosine from 1 mM solution in the presence of 10 mM glucose, 10 mM pyruvate, or 10 mM citrate showed no significant difference from the uptake in the absence of substrate. The essentially complete recovery of a tracer sample of tyrosine during incubation, and its chromatographic identification as pure tyrosine make it clear, however, that catabolism of tyrosine plays an insignificant role in these studies. The intracellular distributions observed, then, are essentially equilibria between uptake and release and not between uptake and metabolism. The tyrosine concentration (1 mM) and the volume of external medium used in most of these experiments was such that no appreciable changes in extracellular concentration were observed during any experiment except where tracer concentrations were used.

**DISCUSSION**

The metabolism of L-tyrosine in this system was investigated in order to assess correctly the factors contributing to the final distribution ratio. The total tyrosine of the system increases during incubation, making balance studies difficult. The extra tyrosine probably arises from proteolysis and the increase could be completely prevented by incubating the diaphragms at 0°C. The essentially complete recovery of a tracer sample of tyrosine after 90 minutes of incubation, and its chromatographic identification as pure tyrosine make it clear, however, that catabolism of tyrosine plays an insignificant role in these studies. The intracellular distributions observed, then, are essentially equilibria between uptake and release and not between uptake and metabolism. The tyrosine concentration (1 mM) and the volume of external medium used in most of these experiments was such that no appreciable changes in extracellular concentration were observed during any experiment except where tracer concentrations were used.

The primary purpose of the studies presented here is to define, insofar as is possible, the mode of entrance of a natural amino acid into the muscle cells of the diaphragm. Two broad categories of transport phenomena are recognized (20–22). The simplest type of transport, diffusion, involves only the forces of thermal agitation and allows no net movement against a concentration gradient. The rate of diffusion depends upon the structure and composition of the barrier, the charge and the size of the penetrant, and the concentration gradient. Diffusion, as a purely physical process, does not involve chemical or enzymatic mediation and should not be directly subject to influences affecting such reactions. The second category, active transport, is best defined as transport against a gradient (20).

It is thought to be enzymatically or carrier-mediated, has a temperature coefficient comparable to that found in most enzymatic reactions, requires energy for its performance or maintenance, is subject to competitive inhibition, enzyme poisons, and saturation kinetics, and generally prevails in systems where diffusion does not play a major role. The process is structurally and sterically specific (21). In some cells the transport of selected compounds, although not concentrative, possesses many of the other properties associated with active transport phenomena. The general term facilitated diffusion has been used to describe such intermediate systems (22).

The entry of tyrosine into cells of the isolated diaphragm exhibits primarily the characteristics of a diffusion process. Thus, the uptake is rapid and reversible, and appears to operate against the gradient only under specialized circumstances. The initial "intracellular uptake" is proportional to the concentration gradient (excluding endogenous) and no saturation phenomena can be observed up to the limits described. Reciprocal plots indicate the infinite V_{max} of a physical process. The volume of distribution is constant at any external concentration if the endogenous tyrosine content is not included in the calculations of the intracellular concentrations. The temperature coefficient is lower than that found in enzymatic reactions and approximates that of a diffusion process. The uptake is insensitive to competitive inhibition by tryptophan, which has been shown to depress the uptake of tyrosine into brain in vivo (6). Wide changes in pH have no significant effect on uptake and changes in the ionic composition of the medium also do not alter characteristics of the entry. Exogenous substrates have no marked effect on the uptake or on the volume of distribution although this might be expected since diaphragm has been shown to possess ample endogenous energy reserves (23). In summary, the entry of tyrosine into diaphragm cells, under these conditions, exhibits (a) no concentrative uptake, (b) a low temperature coefficient, (c) no saturation kinetics (and an infinite V_{max}), (d) insensitivity to changes in pH or ionic composition of the external medium, and (e) no competitive inhibition. These characteristics indicate the lack of an active transport mechanism and strongly implicate a physical process as the mechanism of entry. A similar conclusion has been reached in previous work concerning the entry of tyrosine into cells of liver in vitro (24).

Several lines of evidence lead to the conclusion that the endogenous tyrosine, or an amount approximately equivalent to it,
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The uptake of tyrosine in vitro by intact-diaphragm preparation from fasted rats is characterized by (a) linearity with external concentration, (b) low temperature coefficient, (c) insensitivity to pH change, ionic composition of the medium, or competitive inhibition, and (d) volume of distribution of 80% of the intracellular water. The uptake is rapid and reversible, and equilibrium of uptake and release are essentially complete within 50 minutes.

**SUMMARY**

The data suggest entry of tyrosine into the cell by a stereoselective diffusion process. Kinetic and isotopic data indicate that the endogenous tyrosine, or an amount approximately equivalent to it, is not in diffusion equilibrium with the external medium and does not equilibrate with tyrosine added in vitro during 90-minute incubations.

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**REFERENCES**

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