

## Nature of Insulin Action on Amino Acid Uptake by the Isolated Diaphragm\*

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The use as transport models of amino acids obstructed to metabolism through the absence of  $\alpha$ -hydrogen was suggested in 1955 (1). One of these models,  $\alpha$ -aminoisobutyric acid, shows many of the characteristics of the ordinary amino acids in its transport (2-4). A number of hormonal (5-10) and nutritional (11) effects on its distribution have been reported, including the finding of Kipnis and Noall (12) that its entrance into diaphragm is greatly accelerated by insulin.

More recently, Manchester and Young (13) have reported that of several amino acids tested, the uptake by diaphragm of only glycine and  $\alpha$ -aminoisobutyric acid is accelerated by insulin. This finding raised a question about the general validity of this and similar amino acids as transport models. The entry of  $\alpha$ -aminoisobutyric acid into the isolated diaphragm stops at a point where the apparent distribution ratio is about unity. The entry accordingly could conceivably occur by simple diffusion. When insulin is added, however,  $\alpha$ -aminoisobutyric acid continues to enter the tissue until the level calculated for the tissue water is about two or three times that in the external medium. Apparent gradients are also produced for diaphragm and other muscle in the intact animal. Such apparent uphill migration of amino acids has not been formally shown concentrative for muscle, but by analogy with other tissues we may regard this as likely. Furthermore, it is unlikely that the mode of  $\alpha$ -aminoisobutyric acid transport is entirely changed on addition of insulin. More likely, a mediated transport is uniformly present which operates more actively as soon as insulin is added.

The intestinal transport of this amino acid illustrates the predicted behavior (4). Only under special conditions does this transport lead to accumulation of the amino acid in the intestinal tissue and to the creation of a gradient in favor of the solution on the serosal side. But even under conditions not permitting uphill transport,  $\alpha$ -aminoisobutyric acid moves rapidly across the intestinal wall by a process showing similar sensitivity to competition by other amino acids. Only when accumulation by the tissue and into the serosal compartment occurs is sensitivity to 2,4-dinitrophenol seen.

The present study considers the question whether such model amino acids gain access to the diaphragm atypically, and what kind of an entry process insulin modifies.

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### EXPERIMENTAL PROCEDURE

Female rats weighing from 70 to 100 g and fed on commercial diets were anesthetized by ether.<sup>1</sup> The diaphragms were taken out intact and handled by the technique described by Kipnis and Cori (14). The tissue was incubated in Krebs-Ringer bicarbonate buffer, pH 7.4, to which glucose (final concentration 0.2%) and a labeled amino acid were added. Incubation was carried out aerobically (95% O<sub>2</sub>-5% CO<sub>2</sub>) at 37°. After incubation, the diaphragm was dissected free at its insertions, washed with Krebs-Ringer bicarbonate buffer for 10 seconds and blotted on a filter paper. The wet weight was then measured and the tissue homogenized well in 0.5 ml each of 2% ZnSO<sub>4</sub> and 0.1 N NaOH. After centrifugation, 0.1 ml of the extract was plated on a planchet. The radioactivity was then measured under a gas flow counter, the result being corrected for self-absorption. In some cases, liquid scintillation counting was also used. Extracellular space was determined by inulin in each experiment and was found to be 21 ± 3% of wet weight in the absence of insulin and 23 ± 3% in the presence of insulin. The distribution ratio was calculated as follows: (c.p.m. per ml of intracellular space)/(c.p.m. per ml of incubation medium). In each experiment, 20 to 30 ml of incubation medium were used, so that the concentration of the test amino acid in the medium remained substantially constant during incubation. When the extracellular concentration of amino acid was above 10 mM, the content of sodium chloride in the medium was reduced to keep the osmotic pressure of medium approximately constant.

In almost all cases, the insoluble preparation obtained by trichloroacetic acid extraction of the tissue, followed by methanol-ether washing of the residue, was examined for radioactivity. When diaphragm was incubated with valine, glycine, or methionine, the radioactivity of this proteinaceous material was found to be increased about 1.5 times by the presence of insulin. The finding is similar to the results already reported by some other workers (15, 16). In the presence of the other amino acids examined insulin had no such effect; in several instances, the radioactivity in this precipitate was scarcely significant.

After diaphragm had been incubated with various amino acids, the ZnSO<sub>4</sub>-NaOH extracts were examined by paper chromatography to determine how much of the radioactivity belonged to each free amino acid. More than 90% of radioactivity was shown in each case still to belong to the amino acid originally added.

Oxidized insulin was prepared by the performic acid method

<sup>1</sup> The animals used were the gift of the Upjohn Company.

of Hirs (17). Gelatin (0.2%) was used to prevent the adsorption of insulin to the glassware when insulin was used at concentrations lower than 10 milliunits per ml (18).

### RESULTS

**Effect of Insulin on Uptake of Various Amino Acids**—As may be seen in Fig. 1, after 1 hour of incubation, the distribution ratios of at least eight amino acids are markedly increased by the presence of insulin. These amino acids are AIB,<sup>2</sup> glycine, sarcosine, L- and D-isovaline, L-methionine and L-proline, and a new model amino acid, 1-aminocyclopentane-1-carboxylic acid. In contrast, insulin shows only a slight effect if any on the distribution of seven other amino acids.

**Effect of Insulin on Exodus of Glycine**—After excised diaphragms had been incubated for 2 hours with radioactive glycine (both at 5 mM and at a tracer level), they were transferred into fresh Krebs-Ringer bicarbonate buffer containing a 5 mM or a zero concentration of unlabeled glycine, respectively, and the release of radioactivity from the tissues was then measured. Under these conditions insulin showed no effect on the exodus of glycine.

**Experiments with Oxidized Insulin**—Oxidized insulin had no action on the distribution of AIB, indicating that the action of insulin is inherent in its complete structure and not present in the separated peptide chains. Du Vigneaud *et al.* (19) found that the uptake of glucose also is not enhanced by oxidized insulin.

**Effect of L-Valine and L-Serine on Distribution of AIB**—To determine whether the uptake of AIB occurs by simple diffusion or is chemically mediated, the competitive action of other amino acids was examined. Fig. 2 shows that 5 mM valine or serine substantially slows the entry of  $\alpha$ -aminoisobutyric acid, whether in the absence of insulin (*left*) or the presence of insulin (*right*). The observation period was an hour in the first case, 15 minutes in the latter, these time intervals giving similar degrees of entry. These results show that the entry of AIB uses at least partly the same reaction sites as do the normal amino acids.

**Determination of Apparent Diffusion Constant of  $\alpha$ -Amino-isobutyric Acid Uptake in Presence and Absence of Insulin**—Experiments were then undertaken to determine what type of process is actually enhanced by insulin, one that is subject to saturation or one that fails to show saturation within the concentration range used and therefore follows Fick's law over this range. (The latter process may be physical diffusion, or it also may be mediated but by a reaction site so abundant that it is not saturated.) The following equation may be used to represent the total of these two forms of migration:

$$\frac{dA_c}{dt} = Y + K_D(A_f - A_c) \quad (1)$$

In this formula,  $Y$  is the velocity of uptake by the saturatable process which is taken to be independent of time as well as of intracellular concentration of the amino acid. The experimental justification of this supposition will be described later. When an amino acid is to be transferred only by processes not subject to saturation,  $Y$  is zero. In addition,  $A_f$ , the level in the external

<sup>2</sup> The following abbreviated terms are used: AIB,  $\alpha$ -aminoisobutyric acid; cycloleucine (in the figures), 1-aminocyclopentane-1-carboxylic acid.

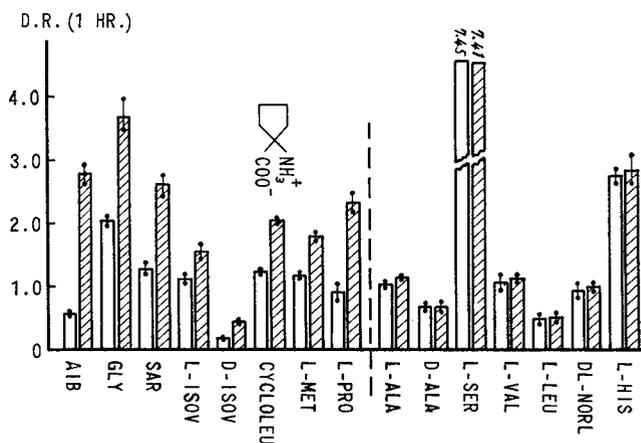


FIG. 1. The effect of insulin on the uptake of various amino acids. Insulin, 0.55 unit per ml, amino acids, 0.5 mM; incubation for 1 hour. The shaded bars show the distribution ratios in the presence of added insulin, the open bars, in its absence. Abbreviations: AIB,  $\alpha$ -aminoisobutyric acid; Sar, sarcosine; IsoV, isovaline; Cycloleu, 1-aminocyclopentane-1-carboxylic acid; Norl, DL-norleucine. The other abbreviations are standard.

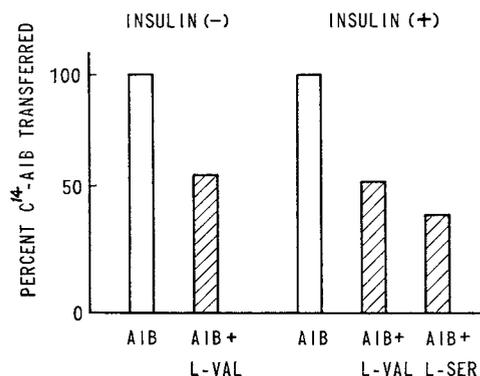


FIG. 2. Inhibitory effect of L-valine and L-serine on the distribution of  $\alpha$ -aminoisobutyric acid (AIB). AIB, 0.5 mM; inhibitors, 5 mM. Incubation, 60 minutes in the absence of insulin, 15 minutes in the presence of insulin.

fluid, remains almost constant during incubation if a large amount of incubation medium is used.  $A_c$  represents the level for the interior of the cells.  $K_D$  is the apparent diffusion constant. By the integration of the above equation, we obtain:

$$\frac{A_c}{A_f} = \frac{Y}{K_D} (1 - e^{-K_D t}) \frac{1}{A_f} + (1 - e^{-K_D t}) \quad (2)$$

If the extracellular concentration of AIB is high, the saturatable component might well be saturated, and in that case,  $Y$  would be very nearly constant. Accordingly, the quantity,  $(1 - e^{-K_D t})$ , is the intercept of the straight line relating  $1/A_f$  and the distribution ratio,  $A_c:A_f$ , at a sufficiently high extracellular concentration.

On the other hand, if the saturatable transfer mechanism is taken to be an enzymatic reaction that can be described by the Michaelis-Menten equation,  $Y$  is given by the following expression:

$$Y = \frac{V_{\max} A_f}{K_m + A_f} \quad (3)$$

If Equation 3 is substituted into Equation 2, we obtain:

$$\frac{A_c}{A_f} = \frac{V_{max}}{K_D} (1 - e^{-K_D t}) \frac{1}{A_f + K_m} + (1 - e^{-K_D t}) \quad (4)$$

In this case, the quantity,  $(1 - e^{-K_D t})$ , is the intercept of the straightline relating  $1/(A_f + K_m)$  again to the distribution ratio  $A_c:A_f$ .

Fig. 3, in which distribution ratios of AIB are plotted against  $1/A_f$ , shows almost identical intercepts for the lines obtained with and without insulin present; that is, insulin has no effect on the diffusion constant of this model amino acid. Approximate values for the diffusion constant can be calculated as 0.22 per hour and 0.20 per hour, respectively, by Equation 2. The distribution ratios obtained after 30 minutes of incubation are also plotted against  $1/A_f$  in Fig. 3. From this result  $K_D$  was determined as 0.23 per hour.

In addition, the saturatable part of the entry of AIB,  $Y$ , in the presence of insulin can also be calculated by substituting the values of  $K_D$  of 0.22 per hour after 1 hour of incubation and of

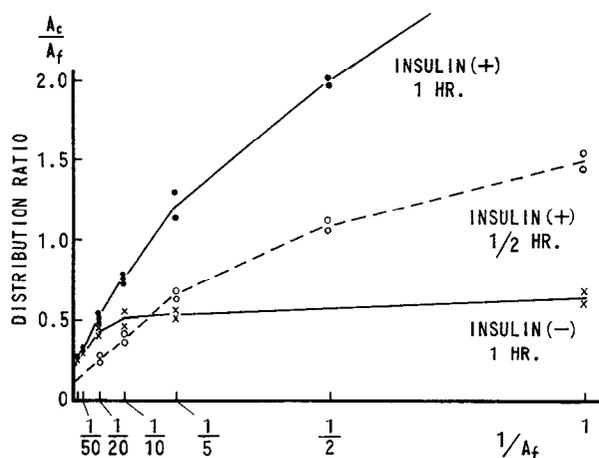


FIG. 3. Relationship of the distribution ratios and the reciprocal of the concentration of  $\alpha$ -aminoisobutyric acid (mM) in external medium. —, results for 1 hour of incubation with and without insulin (0.55 unit per ml); ---,  $\frac{1}{2}$ -hour of incubation.

TABLE I

Constancy of rate of saturatable transport,  $Y$ , during first hour of incubation

The final column shows that saturatable migration is the dominant portion of the uptake in the presence of insulin, although the passive component steadily increases as the level is raised.

AIB in the medium	$Y$		Total entry rate*
	30 minutes incubation	60 minutes incubation	60 minutes incubation
mMoles/liter	$\mu\text{moles/ml/hour}$		$\mu\text{moles/ml/hour}$
1.0	$2.92 \pm 0.15$	$3.01 \pm 0.05$	3.03
2.0	$4.18 \pm 0.15$	$4.00 \pm 0.06$	4.44
5.0	$5.61 \pm 0.26$	$5.69 \pm 0.42$	6.79
10.0	$6.41 \pm 0.10$	$5.98 \pm 0.18$	8.18
20.0	$6.93 \pm 0.21$	$6.96 \pm 0.70$	11.36

\* Total entry rates were calculated by the equation:

$$\text{Total entry} = (Y + K_D S)t$$

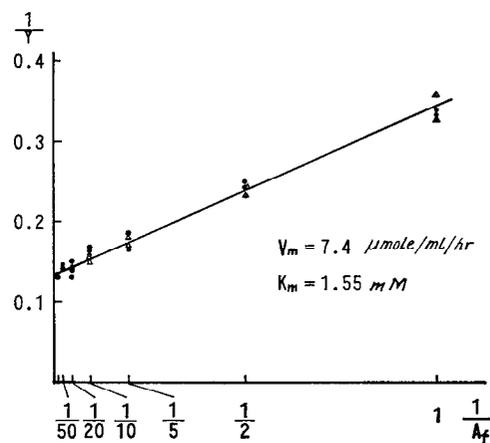


FIG. 4. Applicability of the Michaelis-Menten equation to  $\alpha$ -aminoisobutyric acid uptake by diaphragm. The calculated values of  $Y$  (micromoles per ml of cell water per hour) are plotted against the reciprocal of  $A_f$  (mM) in the presence of insulin. ●, the distribution ratio after 1 hour of incubation; △, that after  $\frac{1}{2}$ -hour of incubation.

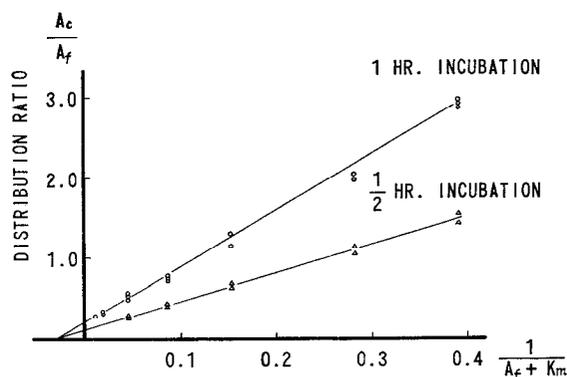


FIG. 5. The distribution ratios plotted against  $1/(A_f + K_m)$  in the presence of insulin (0.55 unit per ml).

0.23 per hour after 30 minutes of incubation, respectively, into the Equation 2. In Table I, comparison of the values of  $Y$  obtained at two different incubation times in the presence of insulin shows that the value of  $Y$  is almost independent of incubation time for at least 1 hour. Fig. 4 shows the relationship between  $Y$  and  $A_f$ , in which  $V_{max}$  and  $K_m$  are estimated as about  $7.4 \mu\text{moles per ml per hour}$  and  $1.55 \text{ mM}$ , respectively. In Fig. 5, distribution ratios are plotted against the quantity  $1/(A_f + K_m)$ ; a straight line relationship results. The intercept  $(1 - e^{-K_D t})$  is almost equal to that obtained in Fig. 3.  $K_D$  is calculated as 0.22 per hour after an hour of incubation and 0.21 per hour after 30 minutes of incubation. These values are almost identical with those obtained in Fig. 3. This result supports the assumption that the saturatable transfer mechanism for AIB can be described by the Michaelis-Menten formulation.

Theoretically, when the distribution ratio is zero, as follows,

$$\frac{V_{max}}{K_D} (1 - e^{-K_D t}) \frac{1}{A_f + K_m} + (1 - e^{-K_D t}) = 0$$

we can obtain by simplification:

$$-\frac{K_D}{V_{max}} = \frac{1}{A_f + K_m} \quad (5)$$

Under these conditions, the quantity  $-K_D/V_{max}$  is independent of incubation time so that both lines, whether for a 1-hour or a 30-minute incubation (in Fig. 3), should give the same intercept on the X axis when distribution ratios are plotted against  $1/(A_f + K_m)$ . Fig. 5 also shows that this expectation is realized. From this intercept on the X axis, the diffusion constant can again be estimated to yield the value 0.21 per hour by using Equation 5. This result also supports the validity of the use above of Equations 1 to 4.

The form of the lower curve in Fig. 3 shows that even in the absence of insulin the transfer of AIB into the diaphragm occurs by a saturatable process, and not simply by free diffusion. Indeed the curvilinear type of relationship between the distribution ratio and  $1/A_f$  (Fig. 3) has almost the same form as the curve obtained in the presence of insulin. The loss in the uptake rate of AIB occasioned by the presence of valine or serine (Fig. 2) undoubtedly represents the almost complete inhibition of the saturatable process. We were not able, however, to calculate  $K_m$  in the case where insulin was absent, since we could not assume that  $Y$  was also independent of time in the absence of insulin, owing to the fact that the distribution ratios at 30 minutes were not high enough to be estimated without large errors.  $K_m$  and  $V_{max}$  in the absence of insulin, however, were roughly estimated as 16 mM and as 8  $\mu$ moles per ml per hour, respectively, assuming that  $Y$  is independent of time and the intracellular concentration.

If this assumption of the independence of the value of  $Y$  with time is valid even in the absence of insulin, a straight line relationship between the distribution ratios and  $1/(A_f + K_m)$  must be obtained by substituting 16 mM as  $K_m$  into the Equation 4. Moreover, the distribution ratios in the absence of insulin must fall on the same straight line obtained in the presence of insulin shown in Fig. 5. Because insulin scarcely has any effect either on the diffusion constant  $K_D$  or on the maximal velocity  $V_{max}$  in Equation 4 the slope and the intercept are the same whether insulin is present or not. In Fig. 6, the distribution ratios in the absence of insulin were plotted against the quantity  $1/(A_f + K_m)$  and found to fall on the same straight line obtained in the presence of insulin. This result suggests that the Equations 1 to 4 also apply for AIB uptake in the absence of insulin.

As a general conclusion we may note that insulin raises the apparent affinity of the saturatable transport site for AIB without changing the maximal velocity of the mediation of transport by this site nor the rate of the migration process not subject to saturation.

*Effect of Insulin at Physiological Levels on Amino Acid Uptake*—The concentration of insulin used for the above experi-

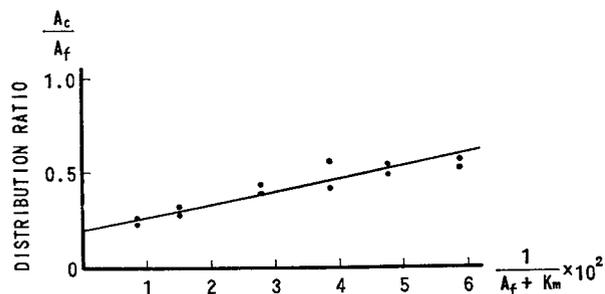


FIG. 6. The distribution ratios after 1 hour of incubation in the absence of insulin plotted against  $1/(A_f + K_m)$ . The line shows the same relationship in the presence of insulin (see Fig. 5).

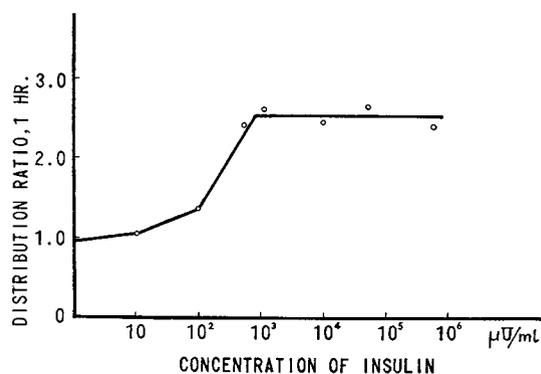


FIG. 7. Effect of insulin at various concentrations on L-proline uptake. L-Proline, 0.5 mM; incubation for 1 hour.

ments was 0.55 unit per ml. This is about 1000 to 2000 times the physiological level. Experiments were then carried out at lower levels of insulin, adding 2 mg of gelatin per ml of the medium to prevent the adsorption of the hormone to the glassware. The added gelatin proved in separate experiments to have no effect on amino acid uptake. Fig. 7 shows that the effect of insulin on L-proline uptake extends to physiological insulin levels. The same has also been shown for AIB uptake.

#### DISCUSSION

Manchester and Young reported that except for glycine the naturally occurring amino acids, so far as they were examined, did not respond to insulin in their rate of entrance into the diaphragm. These authors strongly suggested that AIB has unique characteristics and therefore is not a suitable model amino acid for the study of membrane transport. The above results indicate, however, that the transfer of some natural amino acids, such as L-methionine and L-proline, which they did not examine, is also enhanced by insulin, just as for AIB. In addition, AIB suffers competition with some other amino acids both in the presence and absence of insulin. These findings indicate that the uptake of AIB by isolated diaphragm occurs at least partly by the same mediation as that of the ordinary amino acids.

The finding that insulin accelerates the entrance both of glucose and of AIB into diaphragm has occasionally been interpreted to mean that insulin has simply removed nonspecific barriers to diffusion. The present results show that the action of insulin is not to increase the rate of diffusion, but is to increase the effectiveness with which the mediation functions to produce apparently uphill transport, not only for AIB but for some of the natural amino acids. Presumably, as the affinity of the mediating site for AIB is increased, the rate of uptake exceeds the rate of loss by passive processes so that substantial gradients result.

At the present time we can not identify the feature of amino acid structure that makes certain amino acids more sensitive than others to the insulin action. Symmetry about the  $\alpha$ -carbon is clearly not the only feature. One possibility supported by the present results is that insulin will fail to show an effect on the uptake of an amino acid which has a low apparent  $K_m$  for its active transport. If an amino acid has a value for its apparent  $K_m$  much lower than the concentration used in the external medium, a decrease in the  $K_m$  by insulin might well leave the process still saturated and therefore fail to increase the rate. Unfortunately, the reliable measurement of apparent  $K_m$  values

for the uptake of amino acids of high affinity is difficult, because substantial fractions of these amino acids are metabolized when they are supplied at the low levels required for such experiments.

Our results also indicate that an  $\alpha$ -hydrogen is not necessary to permit apparent uphill transport of amino acids, since 1-aminocyclopentane-1-carboxylic acid and L-isovaline, neither of which have  $\alpha$ -hydrogen, are accumulated by diaphragm even in the absence of insulin. Similar findings have been made with everted intestine where the reality of the uphill transport is not easily questioned (4).

#### SUMMARY

The transfer of amino acids into isolated diaphragm has been investigated with special reference to the uptake of model amino acids and to the action of insulin. The following results were obtained.

1. Insulin enhances the uptake of at least eight amino acids including the naturally occurring amino acids, glycine, L-methionine, and L-proline.
2. Oxidized insulin fails to influence the uptake of the model amino acid,  $\alpha$ -aminoisobutyric acid.
3. L-Valine and L-serine compete with  $\alpha$ -aminoisobutyric acid for uptake by the isolated diaphragm.
4. Insulin raises the apparent affinity of a saturatable site serving for transfer of  $\alpha$ -aminoisobutyric acid without affecting migration processes not subject to saturation.
5. This action of insulin extends from very high to physiological levels.

The origin of the greater sensitivity of the uptake of certain amino acids to insulin stimulation is discussed and the role of  $\alpha$ -hydrogen in amino acid transport is considered.

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