EMERGENCE OF A HIGH AFFINITY TRANSPORT SYSTEM IN ISOLATED HEPATOCYTES FROM FASTING RATS

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The increased capability of the liver to extract gluconeogenic substrates during fasting was investigated at the cellular level by measuring the transport of neutral amino acids in hepatocytes freshly isolated from fed and 48-h-fasted rats. The uptake of 2-α-amino[1-14C]isobutyric acid, a nonmetabolizable analog of alanine, was markedly increased in hepatocytes from fasting rats when measured at 0.1 mM. At the plateau a distribution ratio of 30 to 40 was achieved, compared to 10 in hepatocytes from fed rats. This marked increase was not observed when uptake was measured at 50 mM. Analysis of the relationship between influx and substrate concentration revealed that two independent saturable components contributed to entry of α-aminoisobutyric acid in hepatocytes from fasting rats: 1) a low affinity component (Km = 50 to 70 mM) similar to that observed in cells from fed animals; 2) a high affinity component (Km = 0.6 to 0.8 mM) not observed in cells from fed rats. The transport of α-aminoisobutyric acid occurring through the high affinity component was dependent on Na+, and could be completely inhibited by α-(methylamino)isobutyric acid (Ki = 0.6 mM), the specific substrate for the “A” (alanine preferring) transport system. Ouabain, valinomycin, and gramicidin D partly inhibited α-aminoisobutyric acid transport occurring through this high affinity component. Puromycin and cycloheximide administered in vivo prevented the emergence of the high affinity transport component induced by fasting.

We conclude that the increase in amino acid active transport in hepatocytes from fasting rats results from the emergence of a high affinity transport component which has the properties of a pure “A” system. This system endows the hepatocyte with a high power for concentrating amino acids at low ambient levels, and thus may play an important role in the regulation of gluconeogenesis.

The liver plays a central role in the adaptation to fasting by its increased capacity to extract gluconeogenic substrates (1-4). It is well established that alanine, predominantly synthesized from pyruvate in muscle (3-5) and released in plasma during the early stages of fasting (4, 6, 7), is the preferential gluconeogenic substrate. Nevertheless, it is of prime importance to assess also the capacity of other gluconeogenic substrates during fasting, in particular, α-aminoisobutyric acid. The ability of liver to metabolize large amounts of amino acids is largely dependent upon its transport capability which is rate-limiting for subsequent metabolism. We have investigated this step at the cellular level by measuring the transport of neutral amino acids in hepatocytes isolated from fed and 48-h-fasted rats. In a previous study (8) isolated hepatocytes were shown to take up amino acids through the major transport systems described for eukaryotic cells, namely the “A,” “ASC,” and “L” systems (9). We show here that the capacity of hepatocytes to concentrate AIB, a nonmetabolizable analog of alanine, is strongly increased after fasting. This results essentially from the emergence of a high affinity component of transport which has the properties of a pure “A” system.

MATERIALS AND METHODS

Chemicals—α-Amino[1-14C]isobutyric acid (specific activity = 60 mCi/mmol), 1-aminoacylcyclopentane[1-14C]carboxylic acid (specific activity = 60 mCi/mmol), [3H2O] (specific activity = 1 μCi/ml) and [14C]polyethylene glycol (specific activity = 1 μCi/mg) were purchased from the Radiochemical Centre, Amersham (England); α-methylalaninol[1-14C]isobutyric acid (specific activity = 9 mCi/mmol) was obtained from New England Nuclear. The unlabeled nonmetabolizable amino acids, AIB, N-Me-AIB, and cycloleucine were from Sigma. Valinomycin, gramicidin D, and ATP kit (UV test combination) were from Boehringer, and ouabain from Calbiochem. Other chemicals, most of them listed in a preceding paper (8), were of the best available grade.

Animals—Male Wistar rats (100 to 120 g) were maintained in a constant temperature (23°C) animal room with a fixed 12-h artificial light cycle (8 a.m. to 8 p.m.) for at least 1 week before use. They had free access to laboratory chow (UAR, Villemoisson, Épinay/Orge, France) consisting of (kcal/100 kcal) 63% carbohydrate, 26% protein, and 11% fat. In fasting experiments, rats were deprived of food but had free access to water.

Preparation of Isolated Hepatocytes—Hepatocytes were isolated between 8 and 9 a.m. by collagenase dissociation of the liver as previously described (10). The whole isolation procedure did not exceed 20 min. Hepatocytes were stored at room temperature in Krebs-Ringer bicarbonate buffer (8, 10), gassed with 5% CO2, 95% O2, and used for transport studies within 1 h after isolation.

Measurement of Aqueous Cell Volume and ATP Content—The aqueous cell volume was measured by incubating hepatocytes with 3H2O and 14Cpolyethylene glycol (8). Measured values of intracellular water (H space minus 14C space) were 2.37 ± 0.16 μl/106 cells (n = 12) and 1.41 ± 0.10 μl/106 cells (n = 12) for hepatocytes from fed and 48-h-fasted rats, respectively. The intracellular ATP content was measured by a spectrophotometric UV method, which yielded values of 21.2 ± 0.6 nmol/106 cells (n = 5) and 18.2 ± 0.3 nmol/106 cells (n = 4) for hepatocytes from fed and fasting rats, respectively.

Transport Studies and Expression of Results—Transport assays

The abbreviations and trivial names used are: AIB, α-aminoisobutyric acid; N-Me-AIB, α-(methylamino)isobutyric acid; cycloleucine, 1-aminoacylcyclopentane-1-carboxylic acid.

(Received for publication, July 5, 1978)
were carried out as described previously (8). Experiments were repeated at least twice and each experimental condition was performed in triplicate. Results have been corrected to account for the viability of the cell suspensions, which was estimated by the trypan blue exclusion method and phase contrast microscopy (8, 10).

The Na\(^+\)-dependent part of transport was determined by subtracting, at each substrate concentration, the values obtained in a Na\(^+\)-free medium from total velocities measured in the presence of Na\(^+\) (8). Results relating the Na\(^+\)-dependent velocity (v) to substrate concentration (S) were plotted as v against v/S. When curvilinear plots were obtained the assumption was made that two independent Michaelis-Menten components contributed to transport. Data were submitted to computer analysis to obtain the fit of experimental points and the values of kinetic parameters. The mathematical method used to fit the data involved a least square analysis of the theoretical equation of the hyperbola v against v/S, which describes a transport process where two independent carriers transport the same substrate.

RESULTS

**Time Course of AIB Uptake**—The uptake of AIB by hepatocytes from fed and 48-h-fasted rats was studied with AIB at 0.1 mM and 50 mM, which represent the two extreme concentrations used in subsequent kinetic analysis. Fig. 1 (left panel) shows that the uptake of 0.1 mM AIB by fasting rat hepatocytes increased linearly with time for only 4 min, to reach a plateau after 20 min (distribution ratio, R = 30 to 40). In hepatocytes from fed rats, the uptake increased linearly with time for about 20 min (Fig. 1, left), and the plateau (R = 6 to 10) was not reached before 90 min (not shown). This marked increase in AIB uptake by hepatocytes from fasting rats was not observed with AIB at 50 mM (Fig. 1, right).

**Time Course of AIB Efflux**—The fact that a shorter time was required to reach a plateau in hepatocytes from fasting rats suggested that the rate of exodus might also be altered. Fig. 2 shows the fractional efflux of AIB from hepatocytes of fed and fasting rats preloaded with AIB under conditions where a low intracellular concentration (≈0.5 mM) was achieved. A semilogarithmic plot of the data yielded straight lines (Fig. 2), indicating that efflux processes followed first order kinetics for both types of cells. However, a higher rate of exodus was observed with hepatocytes from fasting rats (t\(_{1/2}\) = 8 min) than with hepatocytes from fed rats (t\(_{1/2}\) = 50 min). Although not shown, no significant difference was observed in the rate of exodus when both types of cells contained a high intracellular concentration of AIB (25 to 30 mM). Therefore, it appears that the increase in AIB transport which is observed in hepatocytes from fasting rats at low substrate concentration involves process(es) operative for influx and efflux.

**Kinetic Analysis of Influx in Hepatocytes from Fed and Fasting Rats**—Fig. 3 shows the dependence of transport on substrate concentration for AIB (Fig. 3, left) and N-Me-AIB, the specific substrate for the "A" (alanine preferring) transport system (9) (Fig. 3, right), in hepatocytes from fed and fasting rats. For fed controls, a plot of the initial velocity of the Na\(^+\)-dependent transport, v, against v/S, was linear over the entire range of substrate concentrations tested for AIB and N-Me-AIB. This indicates that amino acid transport in hepatocytes from fed rats is mediated either by a homogeneous family of carriers, or by several types of carriers that have a similar affinity for the substrate. In hepatocytes from fasting rats, in contrast, the plots were curvilinear (Fig. 3). This can be explained either by cooperative interactions or more likely by the contribution of several independent families of carriers with different affinities, as described for amino acid transport in embryonic heart cells (11). The goodness of the fit observed between the experimental data and theoretical model which assumes that only two independent components participate in transport, supports the latter hypothesis. Table I gives the values of kinetic parameters of the transport components derived from plots of v against v/S (Fig. 3). The resolution of experimental plots for AIB and N-Me-AIB transport in fasting
Amino Acid Transport in Hepatocytes from Fasting Rats

FIG. 3. Concentration dependence of AIB and N-Me-AIB influx. Hepatocytes isolated from fed and 48-h-fasted rats were incubated for 4 min in Na+-containing medium and in Na+-free medium. The range of substrate concentrations tested was 0.1 to 50 mM for AIB, and 0.05 to 50 mM for N-Me AIB. The Na+-dependent component of transport was obtained by subtracting, at each substrate concentration, the Na+-insensitive part from total. Solid lines relating the variation of initial velocity (v) to the ratio of velocity to substrate concentration (v/S) were drawn according to the fitting of the data obtained by computer analysis (see "Materials and Methods"). Dotted lines represent the two Michaelis-Menten components obtained after resolution of curvilinear plots by computer analysis. Each point is the mean of triplicate determinations.

TABLE I

Kinetic parameters of AIB and N-Me-AIB transport in hepatocytes from fed and 48-h-fasted rats

Values of $K_m$ (mM) and $V_{max}$ (nmol/10^6 cells/min) were obtained from experimental plots as shown in Fig. 3, and represent means ± S.E. of n experiments. Systems I and II refer to the low and high affinity components of transport, respectively.

| Amino acid  | Fed System I | Fed System II | Fasted System I | Fasted System II |
|-------------|--------------|---------------|-----------------|------------------|
|             | $K_m$ (mM)   | $V_{max}$     | $K_m$ (mM)      | $V_{max}$       |
| AIB         | 60.7 ± 7.4   | 40.7 ± 3.1    | 52.9 ± 5.4      | 46.6 ± 3.4      |
| N-Me-AIB    | 13.5 ± 1.7   | 18.0 ± 4.1    | 14.3 ± 3.8      | 19.7 ± 2.2      |

rat hepatocytes into two linear components revealed that a high affinity-low capacity and a low affinity-high capacity components contribute to the total, saturable transport. From the values of kinetic parameters, it can be calculated that at low substrate concentrations the high affinity component that operates in fasting rat hepatocytes contributes 75% (at 1 mM AIB) to 85% (at 0.1 mM AIB) of amino acid entry, and thus accounts for the increased accumulation of AIB in hepatocytes from fasting rats compared to cells from fed controls. It should also be noted that, for both AIB and N-Me-AIB, the kinetic parameters of the low affinity transport component in fasting rat hepatocytes were similar to those of the single component that operates in fed rat hepatocytes (Table I).

Effect of Osmolarity on the High Affinity Transport Component—To exclude the possibility that the adsorption of AIB to some cellular component(s) might have accounted for the increased accumulation observed in fasting rat hepatocytes at low substrate concentration, the influx of AIB (at 0.1 mM) was measured in media of varying osmolarities. Fig. 4 shows the relationship between the external concentration of Na+ and the influx of AIB measured at 0.1 mM, a condition in which AIB transport occurs predominantly through the high affinity component. The linear relationship found between v and v/[Na+] indicates a first order dependence of this component on Na+ concentration. The apparent $K_m$ for Na+ ($\approx$60 mM) was analogous to that of the low affinity component of transport present in cells from fed rats (8).

Fig. 5 depicts the effect of Na+ on the kinetic parameters of AIB influx in hepatocytes from fed rats (8), a Na+-independent component of AIB entry was also found in cells from fasting rats. However, this component accounted for only 10 to 15% of total transport (not shown). Fig. 5 shows the relationship between the external concentration of Na+ and the influx of AIB measured at 0.1 mM, a condition in which AIB transport occurs predominantly through the high affinity component. The linear relationship found between v and v/[Na+] indicates a first order dependence of this component on Na+ concentration. The apparent $K_m$ for Na+ ($\approx$60 mM) was analogous to that of the low affinity component of transport present in cells from fed rats (8).
Amino Acid Transport in Hepatocytes from Fasting Rats

The dependence of AIB influx on substrate concentration in hepatocytes from fasting rats was measured at different temperatures and the kinetic parameters of the high affinity transport component were determined. The apparent $K_m$ was not modified, as suggested by the parallelism of the experimental plots (Fig. 7). In contrast, the $V_{max}$ largely decreased when the temperature was lowered from 37-17°C. The activation energy is 16 kcal/mol, calculated from the Arrhenius plot (Fig. 7, inset), is within the range of the values reported for carrier-mediated, active transport processes (14) and is similar to that determined for the low affinity, saturable component of AIB transport operating in fed rat hepatocytes (8).

Inhibition of AIB Transport Occurring through the High Affinity Component in Fasting Rat Hepatocytes—In hepatocytes from fed rats, AIB entry occurs through two $Na^+$-dependent saturable systems, the “A” and “ASC” systems (8). In hepatocytes from fasting rats, the $Na^+$-dependent AIB influx through the high affinity component could be completely inhibited by N-Me-AIB (Fig. 8). Kinetic analysis of competition experiments following the method of Inui and Christensen (15) indicated that N-Me-AIB and l-alanine behave as competitive inhibitors. The $K_i$ for N-Me-AIB (0.4 mM) closely agrees with the $K_m$ (Table I). The d stereoisomer of alanine had no effect (Fig. 8).

The relative ability of natural amino acids to compete for AIB influx is shown in Table II. In hepatocytes from fasting rats, the most potent inhibitors of the high affinity component of AIB transport were alanine, methionine, serine, glycine, and proline. These amino acids were also found to be the most effective in inhibiting AIB influx in hepatocytes from fed rats. However, the extent of inhibition was consistently lower than that observed in hepatocytes from fasting rats (Table II). This is in keeping with the fact that AIB influx occurs through a component with a much higher affinity in hepatocytes from fasting rats than in cells from fed controls.

Cycloleucine Transport in Hepatocytes from Fed and Fasting Rats—To investigate the effect of fasting on the “L” system, the influx of cycloleucine was measured under conditions where the transport of this nonmetabolizable amino acid

Effect of Temperature on the High Affinity Component of

![Figure 5](image-url)  
Fig. 5. Dependence of AIB transport occurring through the high affinity component on external $Na^+$ concentration. The initial rate of 0.1 mM [14C]AIB uptake in hepatocytes from 48-h-fasted rats was determined in the presence of varying $Na^+$ concentrations. Isosomolarity was maintained by replacing $Na^+$ by choline. Inset, $Na^+$-dependent part of AIB transport plotted against the ratio of velocity to $Na^+$ concentration. Each point is the mean of triplicate determinations.

![Figure 6](image-url)  
Fig. 6. Effect of $Na^+$ on the substrate concentration dependence of AIB transport in hepatocytes from 48-h-fasted rats. The relationship between AIB influx and external AIB concentration was studied as described in the legend to Fig. 3, in media containing 40, 60, or 120 mM $Na^+$. Osmolarity was kept constant by replacing $Na^+$ by choline. Inset, values of kinetic parameters for the two transport components obtained by resolution of curvilinear plots; System I and II refer to the low and high affinity transport components, respectively.

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Fig. 8. Inhibition of AIB transport occurring through the high affinity component in hepatocytes from 48-h-fasted rats. The initial rate of 0.1 mM [14C]AIB uptake was measured in the absence or presence of increasing concentrations of N-Me-AIB, L-alanine, and p-alanine. The hatched area represents the contribution of the Na+-independent component to total transport. Relative velocities are expressed as the percentage of the rate of AIB uptake measured in the absence of unlabeled amino acid (referred to as maximal AIB influx) and represent the mean of triplicate determinations.

TABLE II

Relative ability of various \( \text{L} \)-amino acids to inhibit AIB influx in hepatocytes from fed and 48-h-fasted rats

| Amino acid | Fed 100 | Fasted 100 |
|------------|---------|------------|
| None       | 100     | 100        |
| N-Me-AIB   | 38      | 11         |
| Alanine    | 25      | 13         |
| Methionine | 63      | 19         |
| Serine     | 63      | 21         |
| Glycine    | 72      | 32         |
| Proline    | 72      | 34         |
| Phenylalanine | 92 | 63         |
| Arginine   | 75      | 63         |
| Leucine    | 85      | 68         |
| Lysine     | 89      | 70         |
| Valine     | 95      | 82         |
| Glutamic acid | 100 | 88         |

analog occurs through the "L" system (16), i.e. in a Na+-free medium, or in a Na+-containing medium in the presence of a large excess of AIB. Table III shows that, in either condition, fasting did not alter the transport of cycloleucine through "L" system.

Effect of Ouabain, Valinomycin, and Gramicidin D on Active Transport in Hepatocytes from Fed and Fasting Rates—The high affinity transport component induced by fasting and the low affinity component operative in hepatocytes from fed and fasting rats are strongly concentrative (Fig 1). To investigate the possible contribution of alkali-metal ion gradients in the energization of transport, the effects of ouabain, valinomycin, and gramicidin D. [14C]AIB was then added to give the final concentrations indicated and incubations were continued for 4 min. The intracellular ATP content and the AIB influx were then determined on triplicate samples. The results are expressed for both parameters as the percentage of the maximal value obtained in the absence of agent and represent the mean ± S.E. of three separate experiments.

TABLE III

Cycloleucine influx in hepatocytes from fed and 48-h-fasted rats

| Incubation medium      | Cycloleucine influx |
|------------------------|---------------------|
|                        | Fed    | Fasted |
| Na+ medium             | 0.22 ± 0.02 | 0.24 ± 0.02 |
| Choline medium         | 0.21 ± 0.01 | 0.19 ± 0.03 |

TABLE IV

Effect of ouabain, valinomycin, and gramicidin D on the relative ATP content and AIB influx in hepatocytes from fed and 48-h-fasted rats

Hepatocytes isolated from fed and 48-h-fasted rats were preincubated for 10 min in the absence or presence of ouabain, valinomycin, and gramicidin D. \( \text{L} \)-AIB was then added to give the final concentrations indicated and incubations were continued for 4 min. The intracellular ATP content and the AIB influx were then determined on triplicate samples. The results are expressed for both parameters as the percentage of the maximal value obtained in the absence of agent and represent the mean ± S.E. of three separate experiments.

![Fig. 9. Effect of puromycin and cycloheximide on AIB transport in hepatocytes from fasting rats.](http://www.jbc.org)
Effect of Puromycin and Cycloheximide on the Emergence of the High Affinity Transport Component in Hepatocytes from Fasting Rats—When rats were deprived of food overnight, the high affinity transport component was already detectable by kinetic studies, even though its relative contribution to total AIB transport was reduced (Fig. 9). Puromycin (100 mg/kg) and to a greater extent cycloheximide (1 mg/kg) largely prevented the appearance of the high affinity transport component. These results suggest that the emergence of the high affinity transport component in hepatocytes from fasting rats is dependent on new protein synthesis.

**DISCUSSION**

Fasting is accompanied by an increased hepatic extraction of glucogenic substrates and particularly amino acids (1-4). This suggests that the transport of amino acids into the hepatocyte is enhanced during fasting. The present study was designed to investigate this process at the cellular level, using suspensions of freshly isolated hepatocytes from fasting and fed rats.

The fact that an increase in the concentrative uptake of AIB was observed only at low concentrations of substrate in hepatocytes from fasting rats, strongly suggested that a change in carrier affinity may have occurred following fasting. This was confirmed by the analysis of the relationship between initial velocity of transport and substrate concentration, which revealed that more than one Michaelis-Menten component contributes to AIB (and N-Me-AIB) transport in hepatocytes from fasting rats. Quantitative treatment of the data using a curve-fitting method and a computer analysis allowed for the characterization of a high affinity transport component in hepatocytes from fasting rats, in addition to a low affinity component analogous to that present in hepatocytes from fed rats. It should be pointed out that, as stressed elsewhere (9), detection of heterogeneity in transport and characterization of its components require measurements of initial velocities over a very broad range of substrate concentrations and a suitable quantitative analysis.

The high affinity component has the properties of a pure "A" system, as demonstrated by the following observations. 1) AIB transport through this component was completely inhibited by N-Me-AIB, the specific substrate of the "A" system (9); 2) transport through this component was strictly inhibited by N-Me-AIB, the specific substrate of the "A" system (9), and a pre-existing carrier of the "A" type observed in hepatocytes from fasting rats. It is also possible that an adaptive regulation involving a derepression mechanism (24) induced by the low concentration of circulating amino acids in the fasting state might be implicated in the emergence of the high affinity transport system.

The results of efflux experiments deserve comment. It has been suggested that the net entry of amino acids into cells occurs mainly through the "A" transport system, whereas exodus involves the "L" system (17, 18). Our results in fasting rat hepatocytes appear to be inconsistent with this assumption since we observed that the rate of exodus was enhanced despite the fact that the activity of system "L" was not altered by fasting. Thus it seems that the carriers of the "A" transport system might be operative for both upward and downward transport.

Fasting represents a situation where a marked decrease in plasma levels of most amino acids (3-5) is concomitant with an important requirement for amino acids in liver. Accordingly, the emergence of a high affinity transport system endows the hepatocyte with a high power for concentrating amino acids at low ambient levels. In addition to their physiological implications, our findings stress the critical importance of the nutritional state of the animal for the study of amino acid transport processes in hepatocytes. It should be pointed out that even a relatively short period of fasting (24 h) can induce the emergence of a high affinity transport system (see Fig. 9) which may be underestimated or even not detected by a classical analysis of the data.

The entry of amino acids into the liver has been shown to be hormonally dependent in contrast to that of other glucogenic precursors (19). It has previously been shown that in isolated hepatocytes only the "A" system of transport is subjected to hormonal regulation (16, 20-22). Fasting is accompanied by changes in the circulating levels of many hormones (for review see Ref. 23). Thus, the plasma levels of glucagon, glucocorticoids, and growth hormone increase whereas that of insulin decreases. These changes may be involved in the emergence of the high affinity system of the "A" type observed in hepatocytes from fasting rats. It is also possible that an adaptive regulation involving a derepression mechanism (24) induced by the low concentration of circulating amino acids in the fasting state might be implicated in the emergence of the high affinity transport system.

**Acknowledgments**—We are indebted to G. Le Cam for skilful technical assistance, to G. Visciano for illustration work, and to J. Duch for secretarial assistance.

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Regulation of amino acid transport in the liver. Emergence of a high affinity transport system in isolated hepatocytes from fasting rats.
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*J. Biol. Chem.* 1979, 254:401-407.

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