DIRECT MEASUREMENT OF ACTIVE TRANSPORT SYSTEMS FOR ALANINE IN FRESHLY ISOLATED RAT LIVER CELLS

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SUMMARY A method for the direct measurement of alanine transport in liver cells has been developed with the use of alanine aminotransferase inhibitors which block the metabolic utilization of alanine. Freshly isolated rat hepatocytes treated with DL-cycloserine or aminooxyacetate are shown to actively accumulate alanine by three neutral amino acid transport systems, analogous to the A, ASC, and L systems of Ehrlich ascites tumor cells. At a medium concentration of 1mM, these systems contribute 60, 20, and 5% respectively to hepatic alanine uptake. Passive influx accounts for the remainder.

Alanine is utilized primarily as a substrate for gluconeogenesis by liver. Amino acid transport, the initial event in hepatic amino acid metabolism, has been studied in hepatic slices (1), perfused liver (2), isolated cells (3), and hepatocytes and hepatoma cells maintained in culture (4-5). However, because alanine is rapidly metabolized, such studies have employed nonmetabolizable amino acids, e.g. α-aminoisobutyric acid (AIB), in order to elucidate this process. In Ehrlich ascites tumor cells, alanine influx is actively mediated by three different transport systems, whereas AIB is actively transported only by the system designated the alanine-preferring or A-system (6). Thus nonmetabolizable amino acids such as AIB may not adequately describe hepatic alanine transport. The studies here described were undertaken to develop a direct method for characterizing alanine transport in suspensions of freshly isolated rat hepatocytes.

In gluconeogenesis, alanine is first converted to pyruvate, catalyzed by alanine aminotransferase (E.C.2.6.1.2.), a pyridoxal phosphate-dependent enzyme. Cycloserine and aminooxyacetate are known to noncompetitively inhibit pyridoxal phosphate-dependent enzymes in liver (7-8). Previous studies have shown that cycloserine inhibits the formation of [^{14}C]-glucose and $^{14}CO_2$ from [^{14}C]-ala-

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nine and leads to hepatic alanine accumulation (9). We have therefore employed these inhibitors to study alanine transport in isolated liver cells.

MATERIALS AND METHODS Male Sprague-Dawley rats, 200-300 g, were fed standard laboratory chow ad lib until 24 h before use. Isolated liver parenchymal cells were prepared by a modification of the procedure of Wagle and Ingebretsen (10). Livers were perfused with calcium-free Hanks solution containing dialyzed bovine serum albumin (Fraction V), 15mg/ml. Collagenase (Worthington Biochemical) was added following 10 min of perfusion. The dispersion process was completed by gentle agitation of the minced tissue at 37° in the same medium now also containing hyaluronidase (Worthington Biochemical). Separated cells were incubated at 37° in Krebs-Henseleit medium containing dialyzed fatty acid-free bovine serum albumin (Sigma Chemical Co.), 25mg/ml. Cells prepared in this manner excluded trypan blue (92-96%), synthesized glucose from numerous substrates at rates comparable to that for perfused liver, and actively transported AIB in a medium containing 142mM sodium. The cellular potassium concentration at the beginning of incubation was 70-90 mEq/l cell water but rose to 130-150 mEq/l by 30 minutes of incubation. Consequently, a 30 minute preincubation was routinely employed before the initiation of all transport studies. DL-cycloserine, (Regis Chemical Co.), 2mM, aminooxyacetate (Sigma Chemical Co.), 2mM, and substitution of 142mM Na^+ with 142mM choline did not alter the ability of the cells to exclude trypan blue in incubations up to 120 minutes.

Measurements of amino acid uptake were performed by the addition of AIB or L-alanine to suspensions of hepatocytes. Where indicated, [^{14}C]-alanine, 0.05 μCi/ml, was added with unlabeled amino acid. At appropriate times, 2ml aliquots of suspension were removed and the cells immediately sedimented. The supernatant was removed, the centrifuge tube was drained, and the excess medium wiped away with absorbent tissue. The cellular pellet was extracted with 2ml of 10% trichloroacetic acid. Pellet amino acid content was determined by measurement of [^{14}C] radioactivity or by enzymatic measurement of alanine after removal of trichloroacetic acid from the extract with ether (11). Glucose was measured enzymatically (12). The conversion of [U-^{14}C]-alanine to [^{14}C]-glucose was measured following conversion of glucose to glucosazone (13).

The intracellular and extracellular water contents of the cell pellets were determined with parallel incubations in a medium containing unlabeled amino acid, ^{3}HOH, 0.15 μCi/ml, and [^{14}C]-inulin, 0.05 μCi/ml. The intracellular water space was determined as the difference between ^{3}HOH and [^{14}C]-inulin spaces. Total pellet water as determined by ^{3}HOH space measurement corresponded closely to the difference between the wet and dry weight of the cell pellet. The intracellular water space was 45-51% of the ^{3}HOH space. Intracellular amino acid concentration was calculated from the total pellet amino acid minus the amount in the extracellular space and expressed as μmoles per ml cell H_2O.

RESULTS AND DISCUSSION Suspensions of isolated liver cells prepared from 24 h fasted rats exhibited linear rates of gluconeogenesis from a medium containing 10mM alanine (Fig 1). Typically, rates of glucose synthesis ranged between 0.30 and 0.40 μmol/g cells/min. These values closely approximate the rate of gluconeogenesis from 10mM alanine by isolated perfused rat liver (14). The addition of 2mM DL-cycloserine inhibited glucose formation from alanine, and rates of gluconeogenesis decreased to less than that observed in the absence of added

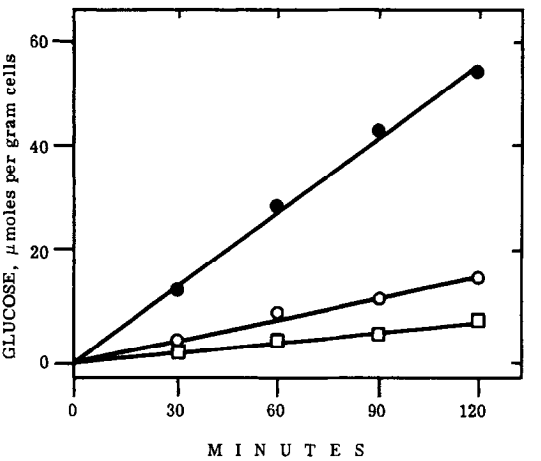

Figure 1 Gluconeogenesis from alanine by freshly isolated rat liver cells. Suspensions of isolated hepatocytes were incubated in Krebs-Henseleit medium containing 10mM L-alanine (●—●), 10mM alanine, and 2mM DL-cycloserine (□—□), or no added substrate (○—○).

substrate (Fig 1). These observations are in agreement with those of Wong et al., who further demonstrated that cycloserine exhibits no discernible effect on the rate of gluconeogenesis from pyruvate, thus localizing the primary action of cycloserine to inhibition of alanine aminotransferase (9). Similar effects of cycloserine were obtained when conversion of [U-^{14}C]-alanine to [^{14}C]-glucose was studied: glucose formation was lowered by approximately 90%. These data are in agreement with previous observations in perfused liver (9).

Brosnan et al. had previously demonstrated hepatocellular alanine accumulation following the in vivo administration of cycloserine (7). The effect of cycloserine in vitro on hepatic alanine content is shown in Figure 2. In a medium containing 10mM alanine, cellular alanine concentration rose transiently in the initial 30 min of incubation but fell thereafter to levels below that of the medium. In contrast, cycloserine-treated cells rapidly attained a cellular concentration 3 times that of the medium. This cellular level of alanine was maintained throughout the 120 min of incubation. With alanine concentrations of less than 10mM in the medium, steady-state intracellular/extracell-

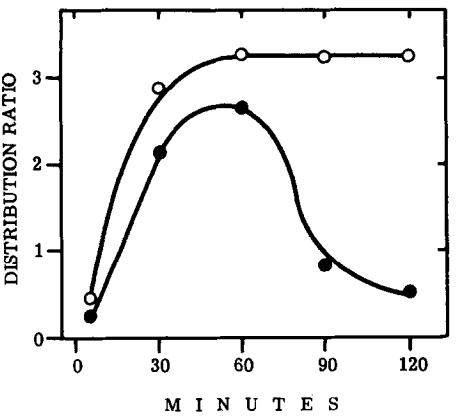

Figure 2 Alanine uptake by isolated rat liver cells. The ratio of intracellular alanine was measured in hepatocytes suspended in a medium containing 10mM alanine (●—●), or 10mM alanine and 2mM DL-cycloserine (○—○).

ular alanine concentration ratios greater than 3 were obtained. Therefore, inhibition of alanine utilization permits the study of the transmembrane flux of this amino acid. Because hepatocellular levels of alanine in freshly isolated hepatocytes approach immeasurably low levels (15), identical results were obtained from the measurement of total alanine and [^{14}C]-alanine. Thus alanine transport in transaminase-blocked cells can be accurately determined by either method.

To rule out the possibility that L-cycloserine might compete with alanine for uptake, studies were performed also with AIB. No measurable effect on AIB transport was observed. Moreover, it was shown that the effect of cycloserine on alanine uptake reached a maximal plateau at 10^{-3}M. Finally, it was shown that alanine transport in cycloserine-treated cells was identical to its uptake in cells exposed to 2mM aminooxyacetate. These properties establish the usefulness of transaminase-blocked cells as a means for studying hepatic alanine uptake.

In Ehrlich ascites tumor cells, alanine uptake is mediated by 3 active transport systems (6). The alanine-preferring (A) and the alanine-serine-cyst-

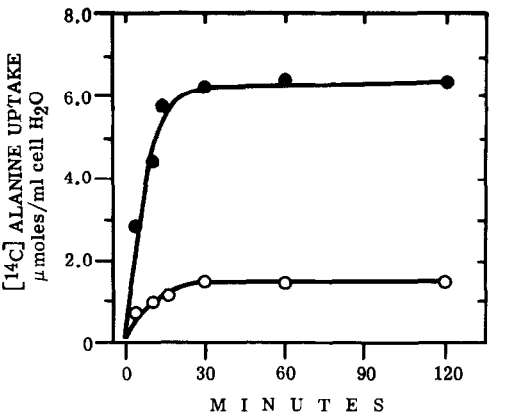

Figure 3 Alanine uptake by isolated rat liver cells. The [^{14}C]-alanine content of hepatocytes was measured in cells suspended in a medium containing 142mM sodium, 1mM L-alanine, and 2mM DL-cycloserine (●—●) or in a medium devoid of sodium containing 142 choline, 1mM L-alanine, and 2mM DL-cycloserine (○—○).

eine (ASC) systems are sodium-dependent while the leucine (L) system is sodium-independent. In these cells, the L system contributes significantly less to net alanine uptake than the A and ASC systems which are of equal importance. Using nonmetabolizable compounds, LeCam and Freychet recently demonstrated that isolated liver cells also exhibit multiple amino acid transport systems (3).

Direct evidence of multiple active transport systems for alanine have now been obtained for hepatocytes. In a medium containing 1mM alanine and 142mM sodium, cycloserine-treated cells accumulated alanine to a concentration 6 times that of the medium (Fig 3). Dinitrophenol, 0.75mM, inhibited this process totally. In Na-free medium containing 142mM choline, intracellular alanine was only 1.5 times that of the medium. Thus the majority of active transport is Na-dependent. The contribution of the L system to alanine uptake was assessed by examining the effect of leucine on sodium-independent alanine accumulation (Fig 4). At a concentration of 7.5mM or greater, leucine inhibited alanine uptake by 25%. The remainder (75%) of the sodium-independent alanine uptake represented passive alanine accumulation. The contribution of the ASC

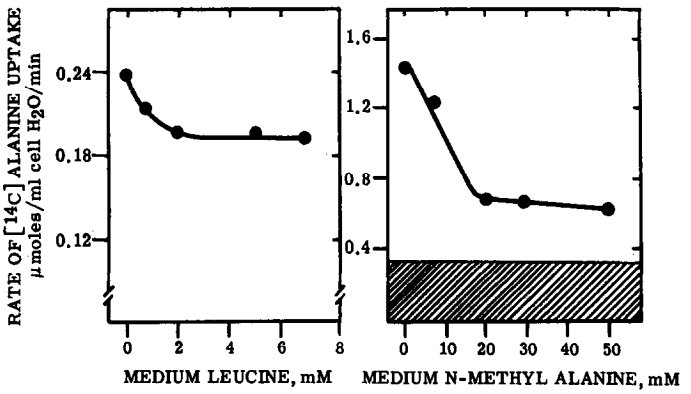

Figure 4 Alanine uptake by isolated liver cells. The initial velocity of [^{14}C]-alanine accumulation was measured in a medium devoid of sodium but containing the indicated concentrations of leucine and 1mM alanine (left panel), or in a medium containing sodium and the indicated concentrations of N-methyl-L-alanine (right panel). The cross-hatched area represents accumulation in sodium-free medium.

system to hepatic alanine uptake was measured using N-methyl-L-alanine, an amino acid solely transported by the A system (Fig 4). In cycloserine-treated cells, 50mM N-methyl-alanine maximally inhibited sodium-dependent alanine uptake by 75%. Thus with 1mM alanine in the medium, the contributions to total hepatic alanine accumulation of the A, ASC, L systems and passive uptake are 60, 20, 5, and 15%, respectively.

This report describes the development of a method for the direct measurement of active alanine uptake in rat liver cells. The studies confirm the recent observations of LeCam and Freychet of multiple neutral amino acid transport systems in hepatocytes (3). Such studies can be performed in freshly isolated hepatocytes since a requirement for culture (4) is not observed. Active alanine transport in liver is mediated by the A, ASC, and L systems in a manner analogous to alanine transport in Ehrlich ascites tumor cells. However, in contrast to Ehrlich cells, the A system is the major effector of hepatic alanine influx.

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