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TRANSPORT OF SOME AMINO ACIDS AND SUGARS IN RAT-LIVER SLICES

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SUMMARY

Intracellular concentration gradients of substrate were obtained when rat-liver slices were incubated in Krebs–Ringer bicarbonate buffer with α-amino[1-^{14}C]isobutyric acid (AIB) under conditions of aerobiosis but not anaerobically. Under aerobic conditions insulin did not affect the uptake of AIB. No concentration gradient was obtained when these slices were incubated in medium containing [1-^{14}C]cycloleucine. No gradients were observed with various radioactive sugars including α- and β-methyl glycosides, 3-*O*-methyl glucose, β-methyl thiogalactoside and potassium galactonate. Various attempts to influence the influx of α-methylglycoside by addition of possible competitive inhibitors, by the use of phlorizin, dinitrophenol and Na^+-free media were without effect. It is concluded that concentration gradients involving an active transport process have been demonstrated by AIB but there was no evidence to confirm the presence of any active transport mechanism for sugars.

INTRODUCTION

The rat-liver slice has been used extensively in studies on glucose metabolism[1] but little information is available concerning the active transport of amino acids and sugars in this system. *In vivo* studies have shown that the concentration of free amino acids in the liver is greater than in the circulating plasma[2] which suggests that concentration gradients for free amino acids can be maintained. *In vivo* studies on the distribution of sugars between the liver and plasma of the rat have generally suggested that no concentration gradient is established for sugars in the liver[3–5] though the recent work of HETENYI AND STUDNEY[6] showed that small gradients for glucose concentration in the liver of the intact rat could be observed and that the concentration gradient was increased in the presence of insulin-induced hypoglycaemia. The high rate of metabolism of amino acids and sugars by the liver complicates investigation of their transport characteristics, and for this reason we have studied the transport of non-metabolised substrates.

Abbreviation: AIB, aminoisobutyric acid.

MATERIAL AND METHODS

α-Amino[1-^{14}C]isobutyric acid (AIB), 1-amino-cyclopentane-1-[^{14}C]carboxylic acid (cycloleucine), uniformly ^{14}C-labelled α-methyl-D-glycoside, uniformly ^{14}C-labelled β-methyl-D-glycoside, 3-*O*-[^{14}C]methyl-D-glucose, β[^{14}C]methyl thiogalactoside and [^{14}C]inulin were obtained from the New England Nuclear Corp. Potassium [*carboxy*-^{14}C]galactonate was prepared by the method of MOORE AND LINK[7]. Non-metabolised sugars without radioactivity were obtained from Mann Research Labs. Incorp. Insulin was obtained from Eli Lilly and Co. (Regular Insulin, Iletin, 40 units/ml). Radioactivity was determined by liquid scintillation counting using Liquifluor (a toluene-based phosphor prepared in concentrated form by New England Nuclear Corp.) and a Packard automatic liquid scintillation counter. Male Sprague–Dawley rats weighing 150–200 g and maintained on rat Purina chow diet were fasted for 24 h before the experiment. The rats were then stunned, decapitated and the liver removed and placed in Krebs–Ringer bicarbonate buffer pH 7.4 at 4°. Slices were then cut on a Stadie–Riggs microtome (0.5 mm thick).

Incubation procedure

All incubations were carried out in triplicate. Freshly prepared liver slices (weighing approx. 50 mg) were incubated in 25-ml conical flasks containing Krebs–Ringer bicarbonate buffer (pH 7.4, 2 ml) and the radioactive substrate dissolved in water. The total volume of solution in which the substrate was added was between 10 and 100 μl depending on the final concentration required. The flasks were routinely gassed with O_2–CO_2 (95:5), sealed with a rubber stopper and incubated at 37° in a Dubnoff shaker. At the end of the incubation the tissue was removed, dipped twice in 0.9 % NaCl for 1 sec at room temperature, blotted, weighed and transferred to 2 ml of distilled water in a centrifuge tube. The tissue was heated in a boiling-water bath for 6 min to extract the radioactive amino acid or sugar. The extract was centrifuged and portions (0.2 ml) of the extract and of the original medium were removed and placed in counting vials. Ethanol (2.8 ml) and Liquifluor (7 ml) were added and the radioactivity of the solution determined.

Total tissue water was determined by the difference between the wet and dry weight of the incubated slice after heating in a vacuum oven at 100° for 18 h. Inulin space was determined by the method described for kidney slices[8] after the addition of 50 μl of [^{14}C]inulin (1.9 mg/ml). Both [^{14}C]AIB and [^{14}C]cycloleucine were added in 10 μl to give a final concentration of 0.065 mM and 0.2 μC/ml. Anaerobiosis was produced by gassing the bicarbonate buffer with 95 % N_2–5 % CO_2 and using this mixture as the gas phase. ^{14}C-Labelled amino acids were introduced into the flasks after a 30-min anaerobic preincubation period. The crystalline insulin, when added, was calculated to give an initial concentration of 0.1 unit/ml or 2.0 units/ml. To maintain the latter high concentration 4 units of insulin were added to the flask at hourly intervals.

The purity of the ^{14}C-labelled amino acids was determined by high-voltage electrophoresis (3 MM Whatman paper, 6.8 % formic acid, 1200 V, 60 mA for 1.5 h at 0°). Virtually all of the ^{14}C in the tissue slices was found to be identical to the unchanged incubated amino acid by the same method.

For a comparison of the efflux of [^{14}C]AIB and [^{14}C]cycloleucine rat-liver slices

were incubated in Krebs–Ringer bicarbonate buffer containing [^{14}C]cycloleucine (0.065 mM) for 60 min in an atmosphere of 95 % O_2–5 %CO_2. Similar slices were incubated in buffer containing [^{14}C]AIB (0.065 mM) for 90 min. After incubation the slices were removed, rinsed in normal saline, blotted and transferred to corresponding flasks containing only Krebs–Ringer bicarbonate buffer (3 ml). These flasks were then gassed with 95 % O_2–5 % CO_2 and the incubation continued. Aliquots of the medium (0.2 ml) were removed at intervals of 4, 8, 12, 16 and 20 min. The radioactivity in these aliquots was determined by scintillation counting. The tissue was removed from the flask after 20 min, rinsed in normal saline, blotted and weighed. The tissue was then heated in a boiling-water bath in water (2 ml) for 6 min and the radioactivity of the tissue-extract ions also determined. The efflux of AIB and cycloleucine was calculated from this data, as described by SCHWARTZMAN, BLAIR AND SEGAL[9].

The chromatographic purity of the sugars used and the nature of the label in the tissue extracts was tested by descending paper chromatography (*tert.*-amyl alcohol–*n*-propanol–water (4:1:1.5, by vol.)[10]. No metabolic products of the ^{14}C-labelled sugars studied were found in the tissue extracts.

Uniformly ^{14}C-labelled α-methyl-D-glucopyranoside and uniformly ^{14}C-labelled β-methyl-D-glucopyranoside were routinely added at 0.2 μC/ml and 0.05 and 0.2 mM, respectively. The glucose concentration used for inhibition studies was 2 mM and the times of incubation were 7.5, 15, 30 and 45 min. Phlorizin (1 mM) was added to the incubation medium and the tissue slices incubated for 2.5, 5 and 10 min to test its effect on sugar transport. Dinitrophenol effects were studied during 10-min incubations at 0.1 mM at α-methyl glucopyranoside concentrations of 0.05, 2 and 20 mM.

The effect of 2-deoxyglucose (20 mM) on the uptake of uniformly ^{14}C-labelled α-methyl glucopyranoside (0.05, 0.5, 5 and 20 mM) by liver slices was also studied. In these experiments a constant amount of radioactivity with increasing amounts of unlabelled material was present.

The uptake of uniformly ^{14}C-labelled α-methyl glucopyranoside (0.05, 0.5, 5 and 20 mM) was measured in buffer in which Na^+ had been replaced by trihydroxyethyl amino methyl ions (Tris)[11] at pH 7.4.

To examine for countercurrent transport of sugars, liver slices were incubated in Krebs–Ringer bicarbonate buffer with 20 mM α-methyl glucopyranoside at 37° for 30 min. Equivalent control slices were incubated without additional sugar in the buffer. At the end of the incubation the tissue was removed, dipped in normal saline, blotted and transferred to flasks containing 0.5 mM of ^{14}C-labelled substrate for a further 5 and 10 min of incubation.

Uptake of potassium [1-^{14}C]*galactonate,* β-[^{14}C]*methyl thiogalactoside and* 3-*O*-[^{14}C]*methyl-glucose by rat-liver slices*

Liver slices were incubated in Krebs–Ringer bicarbonate buffer containing either potassium [1-^{14}C]galactonate (0.5 mM), [^{14}C]Methyl thiolactoside (0.14 mM) or 3-*O*-[^{14}C]methyl glucose (0.1 mM) for 10, 20, 30 and 60 min, respectively. All determinations were carried out in triplicate.

RESULTS

Before calculations can be made of intracellular uptake in a tissue it is necessary to determine the total water content and the fraction of extracellular fluid. The total water content was found to be 71.1 % and the inulin space 21.3 % ± S.D. 0.9. The value for the inulin space remained constant between 10 and 60 min but during longer periods of incubation the inulin space increased to 36 % at 3 h. The value of 21 % is close to that obtained by CAHILL *et al.*[3] when they determined inulin space in the rat liver *in vivo* but they found that the tissue became more permeable to inulin when slices were prepared.

Uptake of AIB and cycloleucine by rat-liver slices

The study of the uptake of [^{14}C]AIB by rat-liver slices showed that although the influx was rather slow it continued in a linear manner for up to 3 h (Fig. 1). The final distribution ratio observed was 4.3 but the uptake was still continuing at that time. On the other hand [^{14}C]cycloleucine reached an equilibrium distribution value in 30 min and did not increase above 1.2 (Fig. 2), which is not significantly different from 1.0 and indicates that no concentration gradient for cycloleucine was being maintained. Radioactive amino acids in the medium and the intracellular form of the amino acid were checked for purity by high-voltage electrophoresis and were found to be at least 98 % pure. The uptake of AIB under anaerobic conditions (Fig. 1) was considerably reduced compared with aerobic conditions and an equilibrium state was rapidly achieved with an isotope distribution ratio of 1.3.

The efflux of AIB and cycloleucine was also studied. Liver slices were preloaded with [^{14}C]AIB for 90 min giving an isotope distribution ratio of 2.5 between the intracellular fluid and the medium, the concentration of AIB in the medium being 0.065 mM. The slices were then transferred into buffer which did not contain any AIB and serial measurements were made of the radioactivity in the medium over a

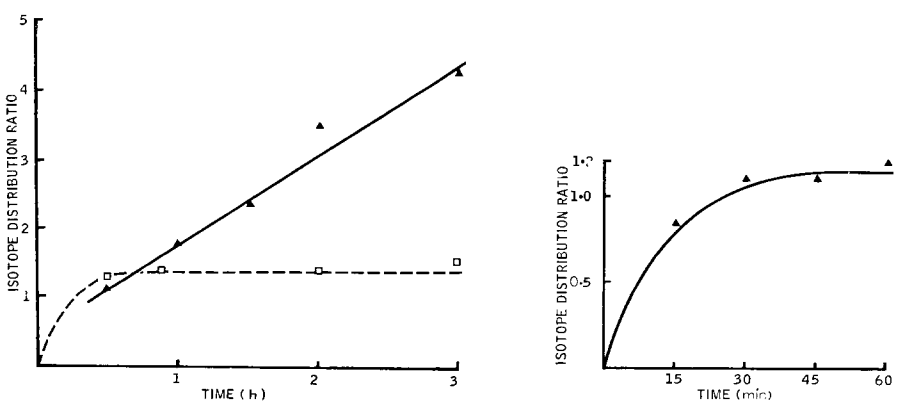

Fig. 1. Uptake of [1-^{14}C]AIB (0.065 mM) in Krebs–Ringer bicarbonate buffer (2 ml, pH 7.4) by rat-liver slices (approx. wt. 50 mg per flask) at 37° incubated under aerobic (▲) and anaerobic (□) conditions. In the latter case the tissue was preincubated in buffer alone for 30 min before addition of [^{14}C]AIB.

Fig. 2. Uptake of [1-^{14}C]cycloleucine (0.065 mM) in Krebs–Ringer bicarbonate buffer (2 ml, pH 7.4) by rat-liver slices at 37° incubated under aerobic conditions.

period of 20 min. These results were then plotted on a logarithmic scale and the calculated value of $t_{0.5}$ was 16.3 min (Fig. 3). Similar measurements were made in which the slices were incubated in a higher concentration of [^{14}C]AIB (0.65 mM) and the efflux of AIB was found to be the same as that at the lower concentration. Liver slices were also preloaded with [^{14}C]cycloleucine (0.065 mM) for 60 min at which time an isotope distribution ratio of 1.3 was obtained. A similar technique was then used for measuring the efflux of cycloleucine and a calculated value for $t_{0.5}$ of 8.6 min was obtained.

Uptake of sugars by rat-liver slices

Simple uptake experiments were carried out with α-methyl and uniformly ^{14}C-labelled β-methyl-D-glucopyranosides into rat-liver slices. Although rapid equilibration of these sugars in the intracellular fluid was only slightly greater than in the medium (Fig. 4) and probably did not indicate that a significant concentration gradient had been obtained. Studies were then carried out to establish whether the transport of these sugars was *via* a carrier-mediated mechanism or by some other mechanism which could be regarded as simple diffusion or non-mediated transport.

In one experiment in which a relatively high concentration of glucose was added to the medium as well as uniformly ^{14}C-labelled β-methyl glycoside, the rate of uptake of the glycoside was not significantly different from that shown in Fig. 4 indicating that competitive inhibition was not taking place.

In further experiments rat-liver slices were incubated in buffer containing α-methyl glycoside (20 mM) and the tissue then transferred to flasks with buffer

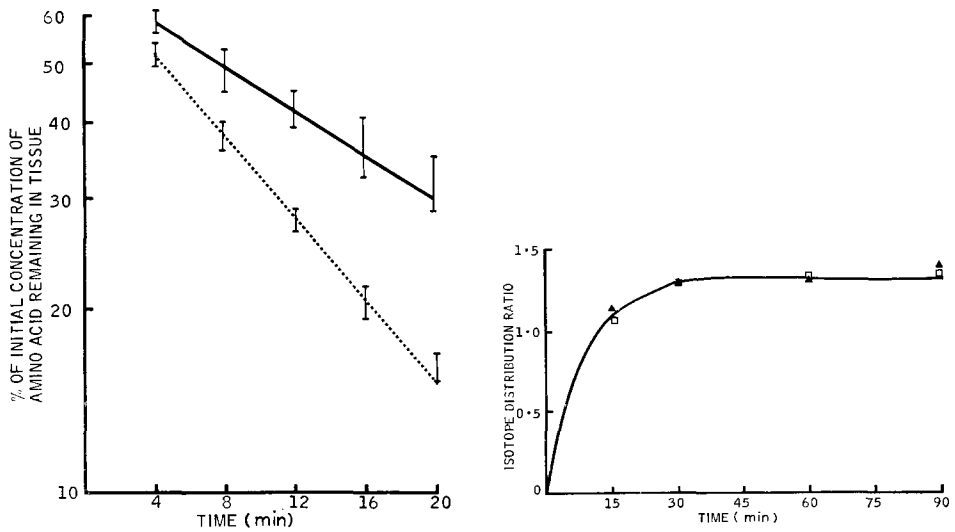

Fig. 3. Efflux of [1-^{14}C]AIB (A) and [1-^{14}C]cycloleucine (B) from rat-liver slices. Liver slices were preloaded with [^{14}C]AIB at both 0.065 and 0.65 mM for 90 min before transferring to separate flasks containing only Krebs–Ringer bicarbonate buffer for the efflux determinations. Liver slices were preloaded with [^{14}C]cycloleucine (0.065 mM) for 60 min.

Fig. 4. Uptake of uniformly ^{14}C-labelled α-methyl-D-glucopyranoside (0.05 mM) (▲) and uniformly ^{14}C-labelled β-methyl-D-glucopyranoside (0.2 mM) (□) in Krebs–Ringer bicarbonate buffer (2 ml, pH 7.4) by rat-liver slices at 37°.

containing uniformly ^{14}C-labelled α-methyl-D-glycoside (0.5 mM). The rate of uptake of this amino acid at 5- and 10-min intervals was not significantly different from that observed in control tissue which had not been incubated with high concentrations of the non-radioactive sugar. These short incubation periods were chosen so that the initial rate of uptake could be studied. A higher rate of uptake in the preloaded tissue would have been evidence for a homo-exchange phenomena, indicating that some carrier mechanism was operating. No such phenomena was observed.

Addition of phlorizin to the medium did not affect the rate of uptake of uniformly α-methyl-D-glycoside whereas in other tissues it has been found to be a potent inhibitor of sugar transport[12]. Experiments were carried out to see if isotope distribution ratio was affected by substrate concentration. At a range of concentration from 0.045 mM to 20 mM the amount of α-methyl glycoside entering the liver slice in 10 min was directly proportional to the substrate concentration, indicating that if an active transport mechanism were operative there was no evidence of saturation up to 20 mM concentration of substrate. 2-Deoxyglucose (20 mM) did not act as an inhibitor of α-methyl glycoside transport at any of these concentrations. Similar experiments were carried out with varying substrate concentrations of α-methyl glycoside in Na^+-free media. Values of the isotope distribution ratio obtained were not significantly different from the values obtained with control incubations in which the usual amount of Na^+ was present. Dinitrophenol (0.1 mM) also did not seem to affect the amount of uniformly ^{14}C-labelled α-methyl-D-glycoside entering the tissue after 10 min, although again the concentration of the substrate was varied over a wide range.

Two other sugars which were not metabolised by the liver under the conditions of the incubation, β-methyl-D-thiogalactoside and 3-*O*-methyl glucose, and one which was only slowly metabolised, potassium galactonate, were also studied. Influx of all these sugars into the slice was fairly rapid and equilibrium was established in 20–30 min. In no case was an isotope distribution ratio greater than 1.5 observed (Fig. 5) and hence in this respect the liver slice was also unable to concentrate these sugars.

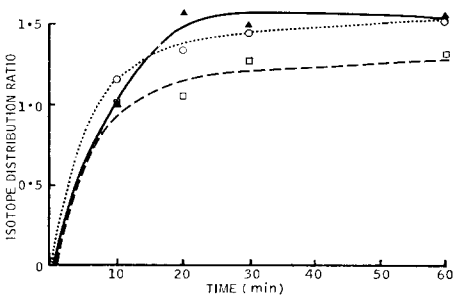

Fig. 5. Uptake of β-[^{14}C]methyl thiogalactoside (0.14 mM) (▲), potassium [1-^{14}C]galactonate (0.5 mM) (□) and 3-*O*-[^{14}C]methyl glucose (0.1 mM) (○) in Krebs–Ringer bicarbonate buffer (2 ml, pH 7.4) by rat-liver slices at 37°.

DISCUSSION

The study of the transport of amino acids and sugars has been carried out *in vitro* in various tissue preparations but little information is available about transport

mechanisms in liver slices. However, this system has been used extensively for metabolic investigations[1]. Some studies have also been carried out on the comparative concentrations of sugars[4] and amino acids[2] in the liver and plasma of various animals *in vivo* and in liver perfusion studies[13]. These studies showed that the intracellular concentration of certain sugars was similar to their concentration in the plasma whereas the concentration of various amino acids was found to be much greater intracellularly than in the plasma. However, the situation was complicated in respect to the naturally occurring sugars, because the rates of metabolism were high and the results obtained were only consistent if the analyses were carried out immediately after the death of the animal and removal of the liver. The experiments of CHAMBERS, GEORG AND BASS[13] with the isolated perfused rat liver showed that the non-metabolised amino acid AIB was concentrated by a factor of 2 in the liver in the basal state. This was increased to 3:1 in the presence of hydrocortisone and 4:1 in the presence of insulin or glucagon. These results were of particular interest from the point of view of the effect of insulin on membrane transport of a non-metabolised amino acid.

Attempts to reproduce such an effect in isolated rat-liver slices were unsuccessful. In the perfused rat-liver studies insulin was infused at a rate of 1 unit per 100 ml of perfusate. In our experiments, when insulin was added to the medium (final concentration 0.1 unit/ml) at the beginning of the incubation period no effect was observed on the uptake of AIB over a period of 3 h. In other experiments insulin (4 units) was added at the beginning of the incubation and a further 4 units at each hourly interval and again there was no effect on AIB uptake over a periode of 3 h. In order to reduce the endogenous insulin to a minimal level the rats were fasted for 24 h before each experiment. The reason for the difference between results of perfusion experiments and the incubation of slices in this respect is not apparent at present.

In a recent communication[14] it was shown that AIB uptake by the rat liver *in vivo* was stimulated by injection of insulin but this did not occur in the adrenalectomized animal. The authors suggested that the apparent stimulation of AIB uptake in the liver by insulin might be mediated by adrenaline and our observation of a lack of stimulation by insulin in the liver-slice system would be consistent with this hypothesis.

In experiments with another non-metabolised amino aicd, cycloleucine, no significant concentration gradient was achieved under aerobic conditions. Efflux studies were carried out with these two amino acids by preloading cells with the radioactive amino acid and then transferring the liver slices to separate flasks containing medium without any added amino acid. Results showed (Fig. 3) that the efflux of cycloleucine expressed as a percentage of the initial tissue concentration of that amino acid was considerably faster than that of AIB. The $t_{0.5}$ for efflux of AIB was found to be the same whether the tissue was loaded by incubation in a medium containing 0.065 or 0.65 M AIB. The shorter $t_{0.5}$ for cycloleucine was therefore not just a function of the difference of the initial concentration of the two amino acids. It is unlikely that the uptake of AIB was just the result of non-specific binding to tissue constituents because efflux of this amino acid occurred in an exponential manner from the slice (Fig. 3) and because under anaerobic conditions no gradient was obtained.

Having demonstrated that the liver-slice system was capable of maintaining a gradient for AIB we then studied the kinetics of transport of various non-metabo-

lised sugars and some allied substances in this system. We were unable to demonstrate any significant concentration gradient for α-methyl glycoside (Fig. 4), β-methyl glycoside, 3-*O*-methyl glucose, β-methyl thiogalactoside or potassium galactonate (Fig. 5). One explanation for this could be that carrier-mediated transport was taking place but that the efflux of these sugars was very high so that no concentration gradient could be achieved. Various experiments were carried out to see if the influx of these sugars could be modified in any way. The rate of uptake of β-methyl glycoside was studied in the presence of high concentrations of glucose (2 mM). If glucose and the glycosides shared any common transport mechanism then inhibition of influx might have been observed, but in fact the initial rates of transport were unaffected by the presence of glucose. Experiments were also carried out in which the slices were preloaded with non-radioactive α-methyl glycoside. Influx of uniformly ^{14}C-labelled α-methyl glycoside was unaffected by this preloading procedure so that no homo-exchange phenomena could be demonstrated. Phlorizin which has been shown to be an inhibitor of sugar transport in the gut[13] and kidney had apparently no effect on the transport of α-methyl glycoside. Experiments with concentrations of α-methyl glycoside in the incubating medium did not affect the influx of sugar so that no saturation of a carrier mechanism could be demonstrated. 2-Deoxyglucose also did not seem to be an inhibitor of α-methyl glycoside transport. The rate of transport of α-methyl glycoside was also found to be unaffected by Na^+-free medium or by dinitrophenol. The outcome of all these experiments with α-methyl glycoside is compatible with the view that there is no carrier-mediated transport mechanism operative in the liver for the concentration of this non-metabolised sugar and this would be compatible with the original observations in intact animals that no concentration gradient for sugars could be obtained. It is perhaps surprising that the liver should differ in this way from other tissues as it is such an important organ for the metabolism of sugars and yet no permeability barriers exist in the membrane of the cell for the control of sugar concentration. Amino acids, on the other hand, do exist at higher concentrations in the liver than in the circulatory plasma[2] and concentration gradients for AIB can be obtained in the perfused liver and in the liver slice.

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