Transport of Methionine and Proline by Rat Liver Slices and the Effect of Certain Hormones

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1. Transport characteristics of L-methionine and L-proline in rat liver slices *in vitro* were studied. 2. Intracellular concentration gradients for methionine were obtained. 3. Methionine uptake was inhibited by iodoacetate, dinitrophenol, Na⁺-free media and also by glycine, lysine, cysteine and dithiothreitol but not by α -aminoisobutyrate. 4. The rate of methionine metabolism in the slice was slow. 5. Puromycin inhibited methionine incorporation into protein, but not methionine uptake. 6. Methionine inhibited the transport of α -aminoisobutyrate but not of cystine. 7. Efflux and exchange diffusion of methionine was studied. 8. Amino acid transport in rat liver slices was not affected by thyroidectomy. 9. Addition of insulin, glucagon, adrenaline or cortisol did not affect the transport of methionine. 10. Addition of 6-N,2'-O-dibutyryl adenosine 3':5'-cyclic monophosphate increased methionine transport after a 120min incubation period in some experiments. 11. Studies of L-proline transport were invalidated because of the rapid evolution of CO₂ from the substrate.

Some studies have been carried out on amino acid transport in rat liver slices *in vitro* (Crawhall & Segal, 1968; Tews & Harper, 1969; Tews *et al.*, 1970; Crawhall & Davis, 1971) to try to identify the transport systems involved and to correlate these with the transport processes taking place in the perfused liver (Chambers *et al.*, 1965, 1968; Mallette *et al.*, 1969*a,b*) and *in vivo* (Scott *et al.*, 1970; Harrison & Christensen, 1971). Some of these studies have involved the use of non-metabolized amino acids. In others, amino acids have been studied that either show low intracellular concentration gradients or are rapidly metabolized in the liver. To find more suitable amino acids for study we have investigated the uptake of methionine and proline by liver slices. Proline was rapidly metabolized, so most of our effort was directed towards methionine transport, in which good intracellular concentrations were obtained with a relatively low rate of metabolism. Methionine transport has been studied in the pancreas by Clayman *et al.* (1968) and in Ehrlich ascites cells by Potashner & Johnstone (1970), but studies in liver slices have not been reported. Tews *et al.* (1970) reported changes in α -aminoisobutyrate transport brought about by various hormonal changes, and some of these have now been investigated for the naturally occurring amino acid methionine.

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Materials and Methods

Chemicals

L-[³⁵S]Methionine and L-[U-¹⁴C]proline were obtained from Amersham-Searle (Arlington Heights, Ill., U.S.A.) and [*carboxy*-¹⁴C]inulin from New England Nuclear Corp. (Boston, Mass., U.S.A.). Amino acids and methylxanthines were obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). Dibutyryl cyclic AMP was purchased from both Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and Schwarz Bioresearch (Orangeburg, N.Y., U.S.A.). Chemicals used in the preparation of Krebs-Ringer buffers came from British Drug Houses (Montreal, P.Q., Canada). Rats were provided by Canadian Breeding Farms, St. Constant, P.Q., Canada. Na⁺-free medium was prepared by substituting tris(hydroxymethyl)aminomethane ions (Tris) for Na⁺ ions (Fox *et al.*, 1964) in the formula for Krebs-Ringer bicarbonate buffer (pH 7.4) (Krebs & Henseleit, 1932).

Slice preparation and incubation procedures

Three male Sprague-Dawley rats, fed on Purina rat chow and weighing 150–200g, were starved for 18h before each experiment, but drinking water was freely available. The rats were killed by decapitation and their livers removed and immersed in oxygenated Krebs-Ringer bicarbonate buffer chilled to 4°C.

Slices (0.5 mm thick) were cut on a Stadie-Riggs microtome (Arthur Thomas, Philadelphia, Pa., U.S.A.) and kept in chilled Krebs-Ringer buffer until incubation. Slices were incubated in 25 ml conical flasks in Krebs-Ringer bicarbonate buffer containing [^{35}S]methionine with or without inhibitors, etc., as described in the Results section (total volume of incubating media was 2 ml). One slice from each of the three rats was incubated in each flask. A total tissue wet weight of 20–50 mg was used. Incubations were carried out in triplicate. Flasks were gassed for 20 s with $\text{O}_2 + \text{CO}_2$ (95:5), then stoppered and incubated at 37°C in a Dubnoff shaker. At the end of the incubation time, the slices were removed, rapidly dipped twice in beakers containing 5 ml of 0.9% NaCl and blotted dry on filter paper. Slices were weighed on a torsion balance (Roller Smith Precision Balances; Federal Pacific Electric Co., Newark, N.J., U.S.A.) and dropped into 2 ml of water in a test tube. These tubes and their contents were immersed in a boiling-water bath for 6 min to liberate amino acids, then cooled, agitated and centrifuged for 5 min at 1000 rev./min (1200g). Radioactivity was determined as follows: 0.1 ml portions of incubation media and of the tissues extracts were added to 10 ml of a scintillation fluid consisting of 4 parts of a toluene dilution (1:25) of Permafluor (Packard Instruments Inc., Downers Grove, Ill., U.S.A.) and 1 part of ethanol (v/v) in glass vials. Samples were counted for radioactivity in a Packard Tri-Carb liquid-scintillation counter. Counting efficiency was optimized for this mixture. Isotope-distribution ratios for amino acid uptake were calculated by the method of Rosenberg *et al.* (1961), and are defined as the ratio:

$$\frac{\text{d.p.m./ml in intracellular fluid}}{\text{d.p.m./ml in extracellular fluid}}$$

Study of methionine metabolism in rat liver slices

Slices were incubated, rinsed, dried and weighed as described above and transferred to water (0.5 ml) in a plastic tube. The slices were homogenized by sonication in a Branson sonifier (Heat Systems-Ultrasonics Inc., Plainview, L.I., N.Y., U.S.A.) fitted with a micro-tip. The instrument was run for 30 s at the maximum energy setting permitted. The aq. 10% (w/v) trichloroacetic acid (0.5 ml) was added to the homogenate, which was mixed, frozen, thawed and centrifuged for 10 min at 2500 rev./min (3000g). The supernatant was removed and extracted with diethyl ether (3 × 1 ml); the aqueous phase was then evaporated to dryness and dissolved in 0.1 M-HCl (0.2 ml). Portions of this solution were subjected to high-voltage electrophoresis (model 2 apparatus; Miles Hivolt Ltd., Shoreham, Sussex, U.K.) in 6.8% (v/v) formic acid at 5 kV for 30 min on Whatman 3 MM paper. The strips containing the radioactive methio-

nine metabolites were cut into pieces 1.3 cm long, and immersed in 10 ml of a toluene dilution of Permafluor (1:25) in scintillation vials and counted for radioactivity. Standards were run alongside the radioactive extracts. The paper containing these was stained with 0.2% ninhydrin in acetone. Each new batch of labelled amino acid was similarly checked for purity by high-voltage electrophoresis.

Measurement of CO_2 evolution during incubation of rat liver slices with L-[^{14}C]proline

Rat liver slices were incubated in conical flasks in Krebs-Ringer bicarbonate buffer containing [^{14}C]proline (2 mM), as described above. The stopper sealing the flask carried a plastic rod terminating in a plastic cup (Kontex Glass, Vineland, N.J., U.S.A.) within the flask. This cup contained Hyamine hydroxide (0.2 ml, 1 M) in methanol to absorb any CO_2 evolved. At the end of the incubation, metaphosphoric acid (1 ml, 2 M) was injected through the stopper into the incubation medium. The flasks were left at room temperature with intermittent shaking for 1 h. The plastic cup was then cut from its supporting rod and dropped into the counting vial containing toluene-based phosphor (Permafluor diluted 1:25 with toluene) (10 ml) and the radioactivity determined in a liquid-scintillation counter (Packard).

Determination of tissue water and inulin spaces

Total tissue water was determined after incubation of liver slices in Krebs-Ringer bicarbonate buffer at 37°C for 60 min. The slices were removed from the flasks, dipped rapidly twice in beakers containing 5 ml of 0.9% NaCl, blotted lightly and weighed. The slices were then dried to constant weight at 100°C *in vacuo* with P_2O_5 as the desiccant. The total tissue water was found to be 3.4 kg/kg dry weight. Inulin space was determined by the method of Rosenberg *et al.* (1962) by using [^{14}C]inulin and was 1.55 kg/kg dry weight after 60 min and 1.75 kg/kg dry weight at 120 min.

Results

Effect of substrate concentration and duration of incubation on methionine uptake

The rate of uptake of [^{35}S]methionine (0.2 mM) by rat liver slices, as measured by the isotope-distribution ratio between the intracellular fluid and the incubation medium, was 3.13 ($\pm$ S.D. 0.9, $n = 11$) at 1 h and remained linear for up to 2 h, when the ratio was 5.9 ($\pm$ S.D. 2.0, $n = 10$). The usual range for isotope-distribution ratio observed in tissue from different flasks in one experiment was typically $\pm 10\%$ of the mean value. Larger standard deviations were observed when results from a series of experiments

Fig. 1. Uptake of L-[^{35}S]methionine (0.2mM) in Krebs-Ringer bicarbonate buffer by rat liver slices at 37°C

The results shown were obtained from six separate experiments in which three separate flasks were used for each time point. Limits of each vertical line represent 1 S.D. about the mean.

carried out in different animals over a period of several months were combined. A composite graph of all these results is shown in Fig. 1. After 2 h the rate of uptake of methionine became very variable.

The rate of uptake of [^{35}S]methionine was measured in a number of experiments over the range of substrate concentrations 0.2–2.0mM with an incubation period of 1 h, and the results were plotted on a double-reciprocal (Lineweaver-Burk) plot of velocity against substrate concentration (Fig. 2). The line of best fit was obtained by the method of linear regression analysis and the intercept of the ordinate and abscissa was calculated from this formula. The results of ten such experiments have been combined to give a calculated K_m of 1.56mM ($\pm$ S.D. 0.32) and a $V_{\max}$ of 0.037 μmol ($\pm$ S.D. 0.009)/g wet wt. of tissue.

Determination of the extent of metabolism of [^{35}S]methionine

Protein-free extracts of the liver slices after incubation in [^{35}S]methionine (0.2mM) for 30, 60 or 90 min were prepared as described in the Materials and Methods section and analysed by high-voltage electrophoresis. There was no evidence of sulphate or taurine formation and all the radioactivity was found in the area corresponding to methionine. The elution pattern of radioactivity of this extract was also studied by automatic amino acid analysis with the Beckman 120 C amino acid analyser. The conditions of the analysis were those described by the manufacturer for the separation of amino acids in

Fig. 2. Rate of transport of L-[^{35}S]methionine at concentrations of 0.2–2mM in Krebs-Ringer bicarbonate buffer by rat liver slices at 37°C plotted as a double-reciprocal plot

Incubation time was 1 h. The points shown are the means of ten separate experiments.

physiological mixtures (Beckman Spinco Technical Bulletin A-TB-015, 1965). This analysis confirmed that at least 85% of the radioactivity was eluted in the methionine region after methionine-uptake experiments in which the incubation was carried out for 1 h. (The amount of radioactivity incorporated into protein was less than 3% of the total radioactivity in the tissue.) This indicated that the determination of total radioactivity was an accurate measure of methionine concentration in the tissue.

Incubation in Na⁺-free medium

Incubation of rat liver slices with [^{35}S]methionine in Na⁺-free medium (Fox *et al.*, 1964) was carried out for 30, 60, 90 and 120 min. In this medium the isotope-distribution ratio did not exceed 1.5, compared with a maximum ratio at 120 min in the Krebs-Ringer bicarbonate buffer of 8.4.

Effect of inhibitors on methionine transport

Similar incubations were carried out in the presence of [^{35}S]methionine (0.2mM) and of sodium iodoacetate at a range of inhibitor concentrations (0.055, 0.55 and 5.5mM) for 60 and 120 min. The lowest concentration of iodoacetate was only weakly inhibitory but at the two higher concentrations inhibition of transport was virtually complete. These results were confirmed in a second experiment with incubations for 30, 60 and 90 min with iodoacetate (0.5mM).

Dinitrophenol (0.4mM) inhibited the uptake of methionine by 85% in experiments carried out for 1 h at methionine substrate concentrations of 0.2, 0.5, 1 and 2mM.

Effect on methionine transport of incubation of rat liver slices in the presence of puromycin

In experiments in which rat liver slices were incubated in Krebs–Ringer bicarbonate buffer with [^{35}S]-methionine (0.2mM) and puromycin (0.18mM) for periods of 30, 60, 90 and 120min, no significant change in isotope-distribution ratio was observed compared with controls incubated in the absence of puromycin. In a subsequent experiment, rat liver slices were incubated with [^{35}S]-methionine (0.2mM) in buffer containing puromycin (0.35mM). A portion of the tissue extract was counted and the remainder treated with an equal volume of 10% (w/v) trichloroacetic acid and the precipitate washed with 10% (w/v) NaCl followed by ether. The precipitate was dissolved by heating at 75°C in Hyamine for 2h. The effect of puromycin at this higher concentration on the uptake of methionine is shown in Table 1. The isotope-distribution ratio for methionine increased by about 40% in the tissue incubated in the presence of medium containing puromycin compared with incubation in non-puromycin-containing medium. Incorporation into protein was decreased to only 10% of the control value, but as reported above the incorporation of methionine into protein is small in relation to the total tissue radioactivity.

Effect of various other amino acids on methionine transport

Rat liver slices were incubated in buffer containing [^{35}S]-methionine (0.2, 0.5, 1 and 2mM) and glycine (20mM) (Table 2). In three separate experiments, inhibition of methionine transport was most significant at a methionine concentration of 0.2mM when

the isotope-distribution ratio was 4.1 after 60min incubation without glycine compared with 2.4 in the presence of glycine. Similar results were obtained with lysine (20mM) as the inhibitor, which inhibited methionine (0.2 and 0.3mM) transport by 30%. No inhibition was observed on addition of α -aminoisobutyric acid (20mM, at methionine concentrations of 0.2, 0.5, 1 and 2mM).

The inhibition of methionine transport was also studied in the presence of cysteine alone, cysteine with dithiothreitol and dithiothreitol alone (Table 3). In the first experiment with cysteine (20mM) and methionine (0.2, 0.5, 1 and 2mM) considerable inhibition was observed, most marked at the lower methionine concentrations. To ensure that the cysteine remained reduced during the aerobic incubation, the experiment was repeated with cysteine (20mM) and dithiothreitol (10mM), and the same degree of inhibition was observed. With addition of dithiothreitol alone inhibition by 75% was observed with methionine (0.2mM) and by 50% with methionine (0.5mM). Apparently this transport system is sensitive to thiol reagents.

Methionine as an inhibitor of transport of other amino acids

Rat liver slices were incubated in the presence of α -aminoisobutyric acid (0.2mM and 1mM) and L-[^{35}S]-cystine (0.2mM and 1mM) respectively for 1h, in paired control experiments in which half the flasks contained L-methionine (20mM). Almost complete inhibition of α -aminoisobutyric acid transport occurred, whereas the transport of L-cystine was not significantly affected.

Table 1. *Effect of puromycin on the uptake of [^{35}S]-methionine by rat liver slices*

Rat liver slices were incubated in Krebs–Ringer bicarbonate buffer containing [^{35}S]-methionine (0.2mM) and puromycin (0.35mM) at 37°C with $\text{O}_2 + \text{CO}_2$ (95:5) atmosphere for the indicated times. Results are the mean of triplicate experiments, each flask containing a liver slice from each of three different rats.

Incubation time (min)	Isotope-distribution ratio	
	Control	+Puromycin
30	1.8	1.8
60	2.8	3.6
90	3.2	4.2
120	3.2	4.5

Table 2. *Inhibition of [^{35}S]-methionine uptake by glycine*

Rat liver slices were incubated in Krebs–Ringer bicarbonate buffer containing [^{35}S]-methionine at the indicated concentrations. Additional flasks contained glycine (20mM) as indicated. Incubations were carried out at 37°C with $\text{O}_2 + \text{CO}_2$ (95:5) atmosphere for 1h. Results are the mean of three different experiments with three flasks for each concentration, each flask containing a liver slice from each of three different rats.

Concentration of methionine (mM)	Isotope-distribution ratio	
	Control	+Glycine
0.2	4.1	2.3
0.5	3.2	2.3
1	2.8	2.6
2	2.0	2.1

Table 3. *Inhibition of [³⁵S]methionine transport by cysteine, cysteine plus dithiothreitol and dithiothreitol alone*

Two separate experiments were carried out, the first with cysteine alone and the second with two inhibitory systems, cysteine+dithiothreitol and dithiothreitol alone. Incubation time was 60 min.

Concentration of methionine (mm)	Isotope-distribution ratio		Isotope-distribution ratio		
	Control	+Cysteine (20mm)	Control	+Cysteine (20mm) +dithiothreitol (10mm)	+Dithiothreitol (10mm)
0.2	3.57	1.63	4.22	1.49	1.75
0.5	3.53	1.58	3.08	1.48	2.33
1	2.79	1.71	2.59	1.36	1.84
2	2.12	1.46			

Electrolyte content of liver slices after incubation

Rat liver slices were prepared in the usual manner and incubated in Krebs–Ringer bicarbonate buffer for periods of 30, 60, 90 and 120 min. The slices were rinsed twice in sucrose solution (5 ml, 0.25 M), blotted and weighed. They were then transferred to water (1 ml) in a test tube, and conc. HNO₃ (0.05 ml) was added to each tube. The tubes were heated for 12 min in a boiling-water bath. The contents were then homogenized by sonication and the tubes centrifuged at 3000g for 5 min.

Analysis for Na⁺ and K⁺ after dilution (1:1) with LiNO₃ solution (30 mm) was done by using a FLMI flame photometer (Bach–Simpson, London, Ont., Canada). It was found that the cation content of the tissue after incubation was approximately 102 μ equiv. of Na⁺/g wet wt. of tissue and 41 μ equiv. of K⁺/g wet wt. of tissue. These values were determined as an average of 12 different determinations in which slices were incubated in four different flasks and for three different periods of time (60, 90, 120 min). The cation content of the tissue was not significantly different between any of these periods of time. McLean (1963) has shown that the K⁺ content of the rat liver slice falls rapidly when placed in 0.9% NaCl at 2°C but rises toward the original value of 350 mequiv. of K⁺/kg dry wt. when incubated for 60 min in Krebs–Ringer phosphate buffer at 38°C. In our experiments the K⁺ content of the slice did not seem to return to the pre-incubation value during incubations of up to 120 min. Analysis of the incubating medium by the same procedure showed it to have a Na⁺ content of 133 mequiv./litre and a K⁺ content of 6 mequiv./litre. The total water content of these slices was 79% of the tissue wet weight and the extracellular space was taken as 36% of the tissue wet weight. The values obtained for the cation content of tissue wet weight were then used to calculate the cation concentration in the intracellular fluid on the assumption that the extracellular space had equilibrated with the incubating medium. The calculated values were 111 mequiv.

of Na⁺/litre and 81 mequiv. of K⁺/litre. The K⁺ content of rat liver slices before incubation was 93 μ equiv./g wet wt. of tissue, which agrees closely with the previously reported value of 97 μ equiv./g wet wt. of tissue (Fenn, 1939) or of 350 mequiv. of K⁺/kg dry wt. (McLean, 1963).

Efflux of methionine from rat liver slices

Rat liver slices were preincubated in Krebs–Ringer bicarbonate buffer containing [³⁵S]methionine (20 mm, approx 1 μ Ci/flask) for 45 min. The slices were then removed, dipped twice in 0.9% NaCl, placed in flasks containing 1 ml of Krebs–Ringer buffer, and regassed (O₂+CO₂, 95:5). The efflux was carried out for 5, 10, 15 and 20 min, and the radioactivity measured in the incubating medium and tissue as described in the previous experiments (Fig. 3). The rate of efflux of methionine which was calculated by the method of Crawhall & Segal (1968) was found to correspond to a decrease of intracellular concentration of methionine to one-half of the starting concentration in 9 min.

Exchange diffusion of methionine

Rat liver slices were preincubated in buffer containing non-radioactive L-methionine (20 mm) for 45 min. The slices were then removed, dipped twice in 0.9% NaCl and transferred to other flasks containing [³⁵S]methionine (0.2 mm). Comparable control slices were preincubated in buffer alone. The second incubations were carried out for 30, 60 and 90 min. The results shown in Table 4 indicate less uptake of methionine in the preloaded slices.

Effect of thyroidectomy on methionine uptake by rat liver slices

Three male Sprague–Dawley rats (4 weeks old, wt. approx. 100 g) out of a litter of six were thyroidectomized. The other three were used as controls and were

Fig. 3. Efflux of L-[^{35}S]methionine from rat liver slices

Slices were preloaded with [^{35}S]methionine (20mM) containing approx. $1\mu\text{Ci}/\text{flask}$ for 45 min at 37°C in Krebs–Ringer bicarbonate buffer. At the end of the preincubation period the slices were removed and placed in fresh buffer (1ml) and the residual radioactivity in the slice was determined after 5, 10, 15 and 20min by the method of Crawhall & Segal (1968).

sham-operated. The rats were maintained on a normal Purina rat-chow diet for 3 weeks and were weighed at intervals. The thyroidectomized animals originally gained weight slowly but subsequently lost weight, and at the time of death they weighed only half as much as their control litter mates. The livers were dissected, weighed and sliced in the usual manner. Liver slices were incubated in flasks containing different concentrations of methionine for 1h such that a Lineweaver–Burk plot of the results could be made. On the basis of uptake related to the weight of the tissue slice the amino acid uptake of liver slices from thyroidectomized animals was identical with that of normal controls. The results obtained by linear regression analysis of the data showed K_m 0.87mM and V_{max} 0.033mmol/min per g wet wt. of tissue for the normal animals and K_m 1.08mM and V_{max} 0.027mmol/min per g wet wt. of tissue for the thyroidectomized animals, these values not being significantly different.

In another experiment with similar thyroidectomized rats the liver slices were incubated with α -aminoisobutyrate, L-cystine, L-lysine or L-leucine (all at 0.2mM) for 1h. No significant difference was found for the uptake of any of these amino acids between liver slices from normal or thyroidectomized animals.

Effect of addition of certain hormones in vitro on the transport of L-methionine

Rat liver slices were incubated for various times in Krebs–Ringer buffer containing [^{35}S]methionine (0.2mM) with addition of various hormones to give

Table 4. Effect of preloading rat liver slices with methionine on the uptake of [^{35}S]methionine

Rat liver slices were preincubated in Krebs–Ringer bicarbonate buffer containing non-radioactive L-methionine (20mM) at 37°C in O_2+CO_2 (95:5) atmosphere for 45 min. The slices were then removed, dipped twice in 0.9% NaCl and transferred to other flasks containing [^{35}S]methionine (0.2mM). Control flasks contained slices preincubated in buffer containing no methionine.

Time of incubation (min)	Isotope-distribution ratio	
	Preloaded	Control
30	1.83	2.36
60	2.67	4.56
90	2.92	5.10

the following concentrations: insulin (Eli Lilly and Co., Indianapolis, Ind., U.S.A., glucagon-free; 120munits/ml or 1.2 units/ml); glucagon (Eli Lilly, crystalline; $2.4\mu\text{M}$); cortisol [5mg dissolved in ethanol (0.4ml) was added to Krebs–Ringer bicarbonate buffer (9.6ml); final concn. 0.16mM]; adrenaline (final concn. 28mM); L-thyroxine (final concn. 1.1mM). Two experiments of each type were carried out with incubation periods of 30, 60 or 90min. In none of these was there any significant difference in amino acid uptake between the control and hormonally treated liver slices.

Effect of dibutyryl adenosine 3':5'-cyclic monophosphate on methionine transport

Rat liver slices were incubated in Krebs–Ringer bicarbonate buffer with L-[^{35}S]methionine (0.2mM) and dibutyryl cyclic AMP (0.5mM). Incubations were carried out for 60 and 120min. No difference was observed in the isotope-distribution ratio obtained after 60min, but in seven out of 15 experiments definite stimulation of uptake was observed at 120min, to the extent of 65% increase of the isotope-distribution ratio compared with the control experiments. In the eight other experiments carried out on separate occasions no significant change of uptake was observed.

Additions of caffeine (1mM) did not increase the effect of dibutyryl cyclic AMP but did, in fact, inhibit the stimulatory effect of cyclic AMP where this was observed. Similarly, no effect was observed on addition of theophylline (1mM). Use of non-esterified cyclic AMP did not cause any stimulation of uptake.

Preincubation of the slices with dibutyryl cyclic AMP for 60min and transfer of the liver slices to a new set of flasks containing dibutyryl cyclic AMP and [^{35}S]methionine did not significantly affect the results.

Fig. 4. Isotope-distribution ratios obtained by incubation of rat liver slices in Krebs–Ringer bicarbonate buffer containing L-[U- 14 C]proline (0.2mM)

Incubations were carried out in triplicate at 37°C in $O_2 + CO_2$ (95:5) atmosphere for the times indicated. The recorded results represent the means of the results obtained.

Neither did the preincubation procedure affect the expected uptake of [35 S]methionine in the control flasks. It is concluded that stimulation of methionine uptake by dibutyl cyclic AMP did occur at 120 min in some experiments but that this was not a consistent effect.

Effect of dibutyl cyclic AMP on transport of other amino acids

Rat liver slices were incubated in flasks containing α -aminoisotopically [14 C]butyrate, L-[1- 14 C]lysine, L-[U- 14 C]proline, L-[U- 14 C]cystine and L-[1- 14 C]tyrosine. Each experiment was carried out in triplicate with paired control flasks with and without dibutyl cyclic AMP (0.5mM). Tissue incubations were carried out for 60min and the isotope-distribution ratio obtained for each amino acid was found to be cystine 5.2, α -aminoisobutyrate and proline 2.0, lysine and tyrosine 1.1 and 1.4 respectively. Addition of dibutyl cyclic AMP had no significant effect on the uptake of any of these amino acids.

Uptake and metabolism of proline by rat liver slices

Rat liver slices were incubated in Krebs–Ringer bicarbonate buffer containing L-[U- 14 C]proline (0.2mM) and a gradient for radioactivity between tissue water and media was established (Fig. 4). In another experiment, by using increasing concentrations for the same length of time (60min) no change of intracellular gradient for radioactivity was observed between concentrations of 0.2 and 10mM. High-voltage electrophoresis showed that after

60 min about 26% of the total radioactivity on electrophoresis was present as a metabolite and after 120 min 35% was present. This was demonstrated by high-voltage electrophoresis in 6.8% (v/v) formic acid buffer in which proline migrated toward the cathode but the metabolite stayed close to the origin, indicating that it had lost its positive charge. It is tentatively suggested that this could be glutamic semialdehyde. A similar study was carried out with incubations in Na^+ -free media. This was associated with a decrease of isotope-distribution ratio at 60 min to 0.85 (control 2.4).

As these results did not explain the failure to obtain an increased intracellular gradient with increase of substrate concentration it was decided to measure the rate of $^{14}CO_2$ formation. Incubations were then carried out in which $^{14}CO_2$ could be trapped as described in the Materials and Methods section for periods of 1 h and 2 h. After 1 h an isotope-distribution ratio of 3 was obtained, but the amount of $^{14}CO_2$ trapped was 2.2 times the total radioactivity in the tissue intracellular fluid and represented 6% of the total radioactivity in the media. After incubation for 2 h the isotope-distribution ratio was decreased to 2.6 and the amount of $^{14}CO_2$ trapped was three times the total radioactivity in the tissue intracellular fluid and represented 10% of the total radioactivity in the medium. The detailed interpretation of these results is complex. Although the proline used was uniformly labelled, electrophoresis never revealed more than 35% of the total tissue radioactivity present as a metabolite, thus indicating that the rest of the proline metabolized intracellularly had all its carbon structure converted into CO_2 .

Discussion

Experimental techniques for measuring amino acid transport in the liver have been carried out with systems *in vivo* (Harrison & Christensen, 1971; Butcher *et al.*, 1971), with systems involving perfusion of the isolated liver (Bass *et al.*, 1970) and with systems using liver slices *in vitro* (Crawhall & Segal, 1968; Tews & Harper, 1969). Such studies of amino acid transport in the liver may be limited by high rates of metabolism of amino acids in that tissue.

The findings presented here indicate that L-methionine fulfils suitable criteria for transport studies *in vitro* in that a gradient for amino acid concentration between the intracellular fluid and extracellular fluid can be obtained. The transport process is energy dependent and the rate of metabolism is relatively slow in relation to the rate of transport.

The transport of methionine *in vitro* has been studied by a few previous investigators. Clayman *et al.* (1968) studied the transport of several amino acids, including methionine, in pancreatic slices obtained

from normal and tumour-bearing mice, and Potashner & Johnstone (1970) studied the transport of methionine in Ehrlich ascites cells. There have been no previous studies of the transport of methionine in rat liver slices but the transport of the non-metabolizable amino acid α -aminoisobutyrate has been studied by Crawhall & Segal (1968) and Tews & Harper (1969). The transport of cystine and cysteine in this system has been studied by Crawhall & Davis (1971) and the effect of various hormones on the transport of α -aminoisobutyrate and cycloleucine in this system has been studied by Tews *et al.* (1970). These various authors have demonstrated that active transport can occur in rat liver slices *in vitro* for certain amino acids. In the present investigation we have shown that methionine can be taken up by rat liver slices with a gradient of at least 5:1 between the intracellular fluid and the extracellular fluid and sometimes with gradients as high as 11:1. The kinetics of this amino acid uptake give a linear relationship on a double-reciprocal (Lineweaver-Burk) plot, from which we have calculated a K_m of 1.56 mM for methionine transport.

Almost complete inhibition of transport occurred in the presence of iodoacetate, dinitrophenol and Na^+ -free media. The rate of metabolism of L-[^{35}S]-methionine in this system was quite slow relative to the rate of uptake of the amino acid. This study of metabolic rate was facilitated by the use of the ^{35}S marker, as the radioactive products of metabolism would not be expected to be volatile and so cannot be lost from the system as can occur with the use of ^{14}C or ^3H labels. Short-term incubations with puromycin successfully inhibited methionine incorporation into newly formed protein but did not affect the rate of uptake of the amino acid. Glycine was an inhibitor of methionine transport, as was lysine to a more limited extent. α -Aminoisobutyrate did not inhibit transport at all in this system. Cysteine was also an inhibitor but so was dithiothreitol, so there is a possibility that cysteine was acting by virtue of its free thiol group rather than as an amino acid and this transport system therefore is thiol sensitive.

The role of methionine as an inhibitor of transport of α -aminoisobutyrate and cystine was also investigated, and whereas the transport of the former was strongly inhibited the latter was not affected. This observation indicated that inhibition of transport by methionine was a specific, not a non-specific, effect. The exchange diffusion of methionine, in which methionine transport in methionine-preloaded slices was measured, did not show the increase in flux that might be expected from the carrier theory of exchange diffusion (Heinz, 1967). In fact it demonstrated a decreased uptake more compatible with the concept of 'trans-inhibition' of Heinz & Durbin (1957), which has been discussed in further detail by Christensen (1972).

The electrolyte content of the slices being incubated in Krebs-Ringer bicarbonate buffer was determined and some loss of the intracellular gradient for K^+ ions was observed which was not corrected during further incubation in buffer at 37°C (cf. McLean, 1963). Previous authors (Tews & Harper, 1969; Judah & Nicholls, 1970) have shown that the K^+ content of the liver slice can be maintained for prolonged incubation periods under conditions similar to those used by us. The only apparent differences between the procedures lay in the breed of rats (Tews & Harper, 1969, used the Holtzman strain), a difference in the technique for the preparation of the slices (Tews & Harper, 1969, used the automatic McIlwain microtome), the lack of additional α -tocopherol in the diet (McLean, 1963; Judah & Nicholls, 1970) and the fact that the electrolyte studies of Tews & Harper (1969) were carried out after incubation in the presence of amino acids whereas ours were not. Van Rossum has shown that the rat liver slice still has the ability to maintain a Na^+ gradient, provided that sufficient intracellular ATP is present (Van Rossum, 1971; Elshove & Van Rossum, 1963).

Judah & Nicholls (1970) have shown that the rate of incorporation of L-[1- ^{14}C]leucine into rat-liver-slice proteins is much diminished if the intracellular K^+ content is depleted. On the other hand analysis of the amino acid pools by automatic amino acid analysis showed that the free amino acid content of the slice was not affected by a reduction of K^+ content and the uptake of L-[1- ^{14}C]leucine was also not affected. It is possible that although amino acid uptake is satisfactory in the presence of a depleted K^+ concentration the sensitivity of the amino acid transport mechanism to hormonal control might be compromised in this situation and might account for some of the insensitivity to hormonal affects to be discussed in the sections below.

Various hormone effects *in vivo* and *in vitro* were also studied. Total thyroidectomy of young growing rats inhibited growth considerably compared with sham-operated animals. At death 3 weeks later the total weights of the livers were considerably less than those of the control animals. However, the transport of methionine appeared to be in no way affected and similar investigations of the transport of α -aminoisobutyric acid, cystine, lysine and leucine showed a similar lack of effect. In certain previous investigations, thyroidectomy increased the uptake of amino acids by the gut mucosa (London & Segal, 1967).

The effect of addition of various hormones *in vitro* on the amino acid transport of methionine was also studied. Addition of insulin, glucagon, cortisol, adrenaline and L-thyroxine did not appear to affect the uptake of methionine in this system during incubations lasting for up to 90 min and at the concentrations of hormone detailed in the present paper. Tews *et al.* (1970) have reported a small stimulation

of uptake of α -aminoisobutyrate in rat liver slices in the presence of glucagon *in vitro* but found that adrenaline was without effect also. In previous studies (Mallette *et al.*, 1969 a,b ; Chambers *et al.*, 1968) it had been shown that both glucagon and adrenaline will stimulate α -aminoisobutyrate uptake by the perfused rat liver, although the effect of adrenaline may be indirect. This effect of glucagon has also been observed in α -aminoisobutyrate-uptake studies in the intact animal (Scott *et al.*, 1970; Harrison & Christensen, 1971).

Cortisol has been shown to increase the hepatic intracellular concentration of cycloleucine in the intact rat (Riggs *et al.*, 1963) and of α -aminoisobutyrate in the liver of the intact mouse (Kaplan & Shimizu, 1962). It has also been shown to stimulate α -aminoisobutyrate uptake by the perfused rat liver (Chambers *et al.*, 1965), but in our experiments addition of cortisol to the rat liver slice *in vitro* did not stimulate methionine uptake.

Because of the relative lack of a direct effect of these hormones *in vitro*, it was decided to investigate the direct effect of dibutyryl cyclic AMP on methionine uptake. Tews *et al.* (1970) observed a consistent increase of α -aminoisobutyrate uptake under the effect of cyclic AMP in rat liver slices and Phang *et al.* (1970) showed that cyclic AMP stimulated the uptake of α -aminoisobutyrate in both rat calvaria and kidney-cortex slices. In our present experiments addition of cyclic AMP significantly increased the uptake of methionine after 120 min incubation, but this effect was not entirely consistent and in some experiments it was not observed. The pattern of results obtained was not affected by preincubation of the slices with cyclic AMP for 60 min before addition of the amino acid as reported in other studies (Tews *et al.*, 1970; Phang *et al.*, 1970). We were also unable to observe stimulation of uptake for a number of other amino acids (α -aminoisobutyrate, lysine, proline, cystine and tyrosine) which were examined.

A separate investigation was carried out on L-proline uptake by rat liver slices in which an intracellular gradient for radioactivity was obtained, but this appeared to be independent of the concentration of amino acid in the incubation medium. Analysis of the intracellular radioactive material by high-voltage electrophoresis showed an unidentified metabolite that had about 25% of the radioactivity of the [^{14}C]proline present in the intracellular fluid. As this did not entirely explain the apparent independence of the intracellular gradient with changing substrate concentration it was decided to measure $^{14}\text{CO}_2$ evolution, and it was found that this was large in relation to the intracellular content of [^{14}C]proline. It is deduced from this that metabolism of proline was occurring at a fast rate but that the metabolites were rapidly converted into CO_2 . Several studies have been reported on L-proline transport in other mammalian

tissues, and in a study of the kinetics of iminoglycine transport in mammalian kidney, Baerlocher *et al.* (1970) found that the rate of CO_2 evolution from L-[^{14}C]proline was dependent on the concentration of substrate and the age of the animal from which the tissue was taken. Mackenzie & Scriver (1971) observed rapid conversion of L-proline into CO_2 by isolated rat kidney glomeruli. Bégin & Scholefield (1965) found that the initial rate of oxidation of L-proline was small in relation to the rate of transport in mouse pancreas *in vitro*, so this effect apparently does not apply to all tissues but does demonstrate that misleading results can be obtained if the possibility of rapid conversion of the substrate into CO_2 without accumulation of intermediate products is not considered.

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