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Restricted Permeability of Rat Liver for Glutamate and Succinate

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1. When rat liver slices were incubated aerobically with [U-^{14}C]glutamate the concentration of ^{14}C within the slices remained lower (about 50%) than in the medium. The maximal concentration of ^{14}C in the liver was reached within minutes. In rat kidney-cortex slices by contrast, ^{14}C reached concentrations more than six times those of the medium. 2. In both liver and kidney ^{14}C appeared in the respiratory CO_2, indicating penetration of glutamate carbon into the mitochondria. In kidney slices the rate of glutamate oxidation per unit weight was about five times that in liver slices. 3. Taking into account the conversion of glutamate into glucose that occurs in the kidney but not in the liver, the flux rates of glutamate through the kidney were calculated to be about 15 times those through the liver when the external glutamate concentration was 5mM. 4. Anaerobically the glutamate concentrations in medium and tissue rapidly became equal in both liver and kidney. Thus the maintenance of concentration gradients depended on the expenditure of energy. 5. [U-^{14}C]Succinate behaved similarly to glutamate. [U-^{14}C]Serine was taken up more rapidly by the kidney than by the liver slices, but the concentrations reached in the liver did not remain below those of the medium. [^{14}C]Urea was distributed evenly between medium and tissue water. 6. Incubation of liver slices with [^{3}H]inulin indicated an extracellular space of liver slices of 26%. 7. When glutamate was generated within liver slices or the perfused liver on addition of oxaloacetate, pyruvate and a source of nitrogen, the concentration of glutamate in the tissue after 1hr. was 70–97 times that in the medium. Thus the exit of glutamate from the liver cell, like its entry, is restricted. This is borne out by measurements of the specific activity of extra- and intra-cellular glutamate on addition of [U-^{14}C]glutamate medium. 8. Liver homogenates removed added glutamate and dicarboxylic acids 20–30 times as fast as did the perfused liver. 9. It is concluded that a major permeability barrier restricts the entry and exit through the outer liver cell membrane.

Experiments on the rate of gluconeogenesis in rat liver from glutamate, aspartate and the intermediates of the tricarboxylic acid cycle indicate that these precursors do not readily penetrate to the site where they are metabolized (Ross, Hems & Krebs, 1967). If added to the perfused liver or to liver slices their rate of removal is slight compared with that of many other metabolites. Experiments reported in this paper provide further evidence for the existence of permeability barriers in rat liver.

EXPERIMENTAL

Tissue slices. Liver and kidneys of Wistar rats were placed immediately after death inside a polythene bag and cooled on ice. Slices were cut freehand with a dry razor blade by the method of Deutsch (1935) and weighed on a torsion balance. They were incubated at 40° in 4ml. of the saline medium of Krebs & de Gasquet (1964) either in Warburg vessels or in 25ml. flasks placed in a Dubnoff (1948) shaking incubator, with O_2 in the gas space. About 100mg. fresh wt. of liver and about 50mg. fresh wt. of kidney were used per vessel. At the end of the incubation the slices were blotted on dry Whatman no. 50 filter paper, reweighed and homogenized with 2ml. of 2% (w/v) $HClO_4$. A 3ml. sample of the medium was deproteinized by the addition of 0·5ml. of 20% (w/v) $HClO_4$. The protein was removed by centrifugation and the supernatant was neutralized with 30% (w/v) KOH to pH7 with 'wide-range' indicator paper. The introduction of an indicator into the sample was avoided since it could interfere with the measurement of radioactivity by scintillation counting. The samples were chilled and the precipitate of $KClO_4$ was removed by centrifugation.

Liver perfusion. Rat liver was perfused with a saline medium containing washed human erythrocytes and

albumin (3·5%, w/v) as described by Hems, Ross, Berry & Krebs (1967).

Radioactive substrates. [U-^{14}C]Glutamate, [U-^{14}C]succinate, [U-^{14}C]serine and [^{14}C]urea were obtained from The Radiochemical Centre, Amersham, Bucks., and [*methoxy*-^{3}H]inulin was from the New England Nuclear Corp., Boston, Mass., U.S.A.

Analytical procedures. Glutamate was determined by the method of Bernt & Bergmeyer (1963), and glutamine as glutamate after hydrolysis at 100° for 5min. in the presence of 2N-H_2SO_4 followed by heating to 100° in the presence of 1N-KOH for 45min. α-Oxoglutarate was determined by the method of Bergmeyer & Bernt (1963); succinate, the sum of L-malate plus fumarate, lactate, pyruvate and glucose were determined as described by Ross *et al.* (1967). Alanine was determined by the method of Williamson, Lopes-Vieira & Walker (1967). Nitrogenous material released by the slices as a result of leakage or disintegration was measured by determining the nitrogen content of the medium as follows. To a sample of medium (0·5ml.) was added 0·5ml. of a mixture of 3 parts of conc. H_2SO_4 and 2 parts of 60% (w/v) $HClO_4$. After the samples had been boiled for 3hr. NH_3 was determined by nesslerization of the diluted sample. The factor 6·3 was used for the conversion of nitrogen values into protein values.

Calculations. Corrections of the analytical values for the tissue content of metabolites were necessary because of loss of tissue material to the medium and because of medium adhering to the slices after blotting. The weight of tissue at the end of the incubation was taken to be the initial weight minus the calculated weight of the tissue loss (nitrogen in the medium multiplied by 6·3). The weight thus calculated was deducted from the measured weight of the slices to obtain the amount of medium adherent to the slices. Radioactivity in the adherent medium was calculated from the analysis of the medium and subtracted from the value obtained for the wet slice.

Determination of metabolic products of [U-^{14}C]*glutamate.* The medium was deproteinized with $HClO_4$ and the supernatant was neutralized with KOH. Of the supernatant 0·1ml. was spotted on Whatman no. 1 paper and treated as described by Gevers & Krebs (1966). The solvent was the 'semi-stench' of Crowley, Moses & Ullrich (1963).

RESULTS

Uptake of [U-^{14}C]*glutamate by rat liver and kidney-cortex slices.* The distribution of glutamate was measured by determining the radioactivity of slices and medium incubated for various periods with [^{14}C]glutamate (Table 1). In the liver the ratio (counts/min./g. of tissue)/(counts/min./ml. of medium) was 0·48–0·52 and showed no significant differences at 15, 30 or 60min. In the kidney the ratios were far above unity (2·30–2·69). Thus glutamate seems to penetrate part of the liver tissue rapidly, but to be excluded from, or kept at a low concentration in, about 50% of the tissue. That some glutamate carbon penetrated the mitochondria of the liver is indicated by the formation of $^{14}CO_2$ (Table 1), but the rate of oxidation was low. From the percentage of added [^{14}C]glutamate recovered as $^{14}CO_2$ (Table 1, last row) and the known rates of respiration, it was calculated that at most 20% of the respiration could have been caused by the combustion of added glutamate. By contrast the kidney rapidly accumulated glutamate to about 2·5 times the external concentration, and the rate of formation of $^{14}CO_2$ was three to four times as high in the kidney as in the liver. Moreover, under the test conditions glutamate is rapidly converted into glucose in kidney cortex (Krebs, Bennett, de Gasquet, Gascoyne & Yoshida, 1963) but not in liver, and since the glucose formed is discharged into the medium more glutamate must have entered the kidney cell than is indicated by the measurement of the distribution of ^{14}C. In 1hr. 6·1% of the added glutamate carbon (1·2μ-

Table 1. *Uptake of* [U-^{14}C]*glutamate by rat liver and kidney-cortex slices*

Liver slices (100mg. fresh wt.) and kidney-cortex slices (50mg.) were incubated in 4ml. of saline medium with O_2 in the gas phase. The initial concentration of the glutamate was 5mM (including L-[U-^{14}C]glutamate with the radioactivity about 2μc/vessel). The CO_2 was collected in duplicate vessels containing 0·5ml. of 0·1N-HCl in a side arm and a small tube with NaOH solution in the centre well. The acid was added at the end of the incubation and 15min. further shaking was allowed for the absorption of CO_2 by the alkali. For other details see the text.

Tissue	Liver			Kidney		
Incubation time (min.)...	15	30	60	15	30	60
10^{-4} × Radioactivity of medium (counts/min./ml.)	22·2	22·4	23·4	22·2	20·9	19·6
10^{-4} × Radioactivity of slices (counts/min./g.)	11·5	10·8	11·9	51·7	50·3	52·9
Ratio $\frac{\text{(counts/min./g. of slices)}}{\text{(counts/min./ml. of medium)}}$	0·52	0·48	0·51	2·33	2·40	2·69
10^{-4} × Radioactivity of CO_2 (counts/min./g. of slices)	11·5	14·7	20·1	33·5	55·6	88·2
% of radioactivity added as glutamate and recovered as CO_2	1·38	1·60	2·46	1·55	3·69	6·10

Table 2. *Early time-course of the uptake of glutamate by rat liver and kidney-cortex slices*

[U-^{14}C]Glutamate (2μc) was added from a side arm after a preliminary incubation of 10min. The medium contained 1mM-glutamate. For other details see Table 1.

Tissue	Liver		Kidney			
Incubation time (min.) ...	2	5	1	2	3	5
$10^{-4}\times$ Radioactivity of medium (counts/min./ml.)	16·4	15·1	15·1	15·0	15·9	14·3
$10^{-4}\times$ Radioactivity of slices (counts/min./g.)	9·2	8·5	8·5	11·6	21·6	28·6
Ratio $\frac{\text{(counts/min./g. of slices)}}{\text{(counts/min./ml. of medium)}}$	0·56	0·56	0·56	0·77	1·36	2·00

Table 3. *Comparison of aerobic and anaerobic uptake of glutamate by rat liver and kidney-cortex slices*

Tissue was incubated as described in the text. The concentration of [U-^{14}C]glutamate was 5mM (radioactivity about 2μc/vessel). Anaerobic conditions were obtained by a stick of yellow phosphorus in the centre well.

Tissue	Liver				Kidney			
Incubation time (min.)...	15	30	15	30	15	30	15	30
Gas	O_2	O_2	N_2	N_2	O_2	O_2	N_2	N_2
Ratio $\frac{\text{(counts/min./g. of slices)}}{\text{(counts/min./ml. of medium)}}$	0·62	0·65	0·81	0·78	2·78	4·02	0·60	0·82

moles) appeared as carbon dioxide. The same amount of kidney converts 3·7μmoles of glutamate into glucose in 1hr. Since twice as much liver as kidney was used in the experiments of Table 1, it follows that the flux of glutamate through the kidney was about 15 times as great as the flux through the liver, and in spite of the rapid utilization by the kidney the tissue accumulated glutamate.

The early time-course of the glutamate uptake by the liver (Table 2) indicates that the glutamate concentration in the tissue reached its maximum level within 1min., whereas in kidney there was a steady rise over the first 5min. (Table 2) followed by a further slow rise throughout 1hr. (Table 1). These findings support the view that there is a rapid penetration of glutamate into some compartments of the liver cell and a restricted entry into others.

As the radioactivity measurements do not distinguish between the added substrates and non-volatile metabolic products they indicate the maximal glutamate concentration. This implies that steady-state concentration gradients of [^{14}C]glutamate might be even less than the results indicate, but after short incubation periods the total quantities of radioactive material other than glutamate must have been small.

When the experiments were carried out anaerobically the radioactivity ratios after 30min. were 0·78–0·87 in the liver and 0·82–0·97 in kidney cortex (Table 3). In these experiments the [^{14}C]-glutamate was added from a side arm after a preincubation of 10min., during which complete anaerobiosis was established by a stick of yellow phosphorus in the centre well of the Warburg vessel. The concentrations stated in Table 3 refer to the whole tissue as opposed to tissue water. Since about 23% of the tissue is composed of solids a ratio about 0·8 would indicate equilibrium between tissue water and medium water. The greater accumulation of glutamate in the liver under anaerobic conditions suggests that the barrier preventing entry of metabolites depends on the expenditure of energy. In the kidney the effect of anaerobiosis is the opposite: the accumulation of glutamate in the tissue against the concentration gradient is abolished and the external and internal concentrations become equal. Hence the concentration of the intracellular glutamate reached in the kidney anaerobically is less than one-quarter of that obtained aerobically.

Analogous differences in the accumulation of glutamate in liver and kidney were found by Friedberg & Greenberg (1947) after intravenous injection: kidney accumulated glutamate whereas liver did not.

Uptake of [U-^{14}C]*succinate and* [U-^{14}C]*serine by*

Table 4. *Uptake of* [U-^{14}C]*succinate by rat liver and kidney-cortex slices*

The experimental conditions were as described for Table 1, except that the final substrate concentration was 1mM.

Tissue	Liver			Kidney		
Incubation time (min.)...	5	15	30	5	15	30
$10^{-4}\times$ Radioactivity of medium (counts/min./ml.)	102	104	103	104	97	94
$10^{-4}\times$ Radioactivity of slices (counts/min./g.)	49	48	60	182	220	308
Ratio $\frac{\text{(counts/min./g. of slices)}}{\text{(counts/min./ml. of medium)}}$	0·48	0·46	0·58	1·75	2·29	3·27

Table 5. *Uptake of* [U-^{14}C]*serine by rat liver and kidney-cortex slices*

The experimental conditions were as described for Table 2.

Tissue	Liver			Kidney		
Incubation time (min.)...	5	15	30	5	15	30
$10^{-4}\times$ Radioactivity of medium (counts/min./ml.)	58	57	57	58	58	58
$10^{-4}\times$ Radioactivity of slices (counts/min./g.)	38	54	59	65	102	135
Ratio $\frac{\text{(counts/min./g. of slices)}}{\text{(counts/min./ml. of medium)}}$	0·66	0·95	1·04	1·12	1·76	2·33

Table 6. *Uptake of* [U-^{14}C]*glutamate and of* [methoxy-^{3}H]*inulin by rat liver slices*

The general experimental conditions were as described for Table 1, except that substrates were 1mM-[U-^{14}C]-glutamate (about 2μc/vessel) and carrier-free [*methoxy*-^{3}H]inulin (about 2μc/vessel).

Incubation time (min.) ...	5	15	30	60
$10^{-4}\times$ ^{14}C radioactivity in medium (counts/min./ml.)	34·3	34·1	31·4	34·3
$10^{-4}\times$ ^{14}C radioactivity in slices (counts/min./g.)	15·7	11·4	16·2	19·7
Ratio $\frac{\text{(^{14}C counts/min./g. of slices)}}{\text{(^{14}C counts/min./ml. of medium)}}$	0·45	0·34	0·52	0·57
$10^{-4}\times$ ^{3}H radioactivity in medium (counts/min./ml.)	124	127	119	131
$10^{-4}\times$ ^{3}H radioactivity in slices (counts/min./g.)	12·9	22·4	30·8	33·2
Ratio $\frac{\text{(^{3}H counts/min./g. of slices)}}{\text{(^{3}H counts/min./ml. of medium)}}$	0·10	0·18	0·26	0·25

rat liver and kidney-cortex slices. Results obtained with succinate (Table 4) were very similar to those obtained with glutamate. The overall concentration of radioactivity in the tissue was calculated to be 60% of that of the medium, whereas in kidney it reached more than 300% of that of the medium, with no allowance made for the fact that the water content of the tissue is about 77% of the liver weight and 81% of the kidney-cortex weight. To reconcile these findings with the observation by Rosenthal (1937) that the oxygen uptake of rat liver slices can be greatly increased by the addition of succinate, it has to be postulated that the rate of penetration into the slice is much lower than the rate of oxidation. This is supported by the fact that the rate of oxidation depends on the thickness of the slices. With serine (Table 5), which is readily metabolized in both tissues, the radioactivity of the slices rose above that of the medium, but more so in kidney cortex than in liver.

Glutamate space and extracellular space. To relate the space available to glutamate or succinate to the 'extracellular' space in the slices, liver and kidney were incubated simultaneously with [^{14}C]-glutamate and [^{3}H]inulin. The uptake of the two radioactive isotopes was determined in the three-channel Beckman scintillation counter adjusted to counting ^{14}C and ^{3}H separately. As shown in Table 6 the concentration of inulin in the liver slices after 30min. was 26% of that of the medium, in good agreement with previous findings (see Rosenberg, Downing & Segal, 1962) that the 'extracellular space' is about 30%. If the concentration of glutamate in the areas into which it penetrated was the same throughout, the calculation indicates that 50–60% of the liver slice was

Table 7. *Uptake of* [^{14}C]*urea by rat liver and kidney-cortex slices*

Slices were incubated as described in the text. The urea concentration was 2mM (radioactivity about 2μc/flask).

Tissue	Liver					Kidney				
Incubation time (min.)	1	2	3	5	15	1	2	3	5	15
Ratio $\frac{\text{(counts/min./g. of slices)}}{\text{(counts/min./ml. of medium)}}$	0·47	0·64	0·71	0·69	0·79	0·63	0·66	0·63	0·77	0·76

Table 8. *Distribution of glutamate (added or generated within the tissue) between rat liver slices and suspending medium*

Slices were incubated as described in the Experimental section.

Expt. no.	Substrates added (all 5mM)	Glutamate in medium (μmoles/ml.)		Glutamate in slices (μmoles/g.)		Ratio glutamate in slices / glutamate in medium	
	Incubation time (min.)	30	60	30	60	30	60
1	None	0·033	0·042	0·47	0·62	14·2	14·8
	Oxaloacetate, pyruvate, NH_4Cl	0·016	0·022	0·89	2·14	56	97
2	None	0·15	0·10	1·93	1·60	13	16
	Oxaloacetate, alanine	0·050	0·041	2·64	2·88	53	70
	Oxaloacetate, pyruvate, NH_4Cl	0·034	0·054	2·70	3·43	79	64
3	L-Glutamate	4·07	4·37	1·93	1·75	0·47	0·40

accessible to glutamate. It follows that the penetration of glutamate was not restricted to the 'extracellular space'. ^{131}I-labelled albumin gave results similar to those of [^{3}H]inulin.

Experiments with [^{14}C]*urea.* The distribution of [^{14}C]urea between slices and medium was investigated because urea freely penetrates the tissue and is not accumulated. As shown in Table 7 the radioactivities in both kidney and liver reached about 80% of those of the medium within 5min. This value is the maximum expected if the tissue water is in equilibrium with the aqueous phase of the medium.

Distribution of glutamate generated within the liver cells. Glutamate readily arises within liver cells when precursors that penetrate into the liver are provided. Precursors of this kind are a mixture of oxaloacetate, pyruvate and a source of nitrogen such as alanine or ammonium chloride. The formation of glutamate from these precursors involves the intramitochondrial formation of α-oxoglutarate by the reactions of the tricarboxylic acid cycle and reductive amination or transamination. On incubation of liver slices with these precursors much of the glutamate formed remained within the slices, so that high concentration gradients were built up (Table 8). The concentration gradient between rat liver and blood plasma *in vivo* is known to be about 15 (Schimassek & Gerok, 1965). The concentration gradients between slice and medium rose on incubation to 70–97 (Table 8) and the tissue concentration of glutamate became much higher than on incubation with 5mM-glutamate.

Parallel observations were made with the perfused rat liver (Table 9). Addition of glutamate to the perfusion medium did not significantly raise the glutamate content of the liver, whereas oxaloacetate plus alanine more than doubled it. In these experiments the substrates were added to the medium to a final concentration of 5mM after a preliminary perfusion period of 38min. and 60min. Later, a sample (about 250mg.) of the liver was clipped off with scissors, placed in 2% (w/v) perchloric acid solution (4ml.) and homogenized. No correction was made for the presence of some perfusion fluid in the liver. Where glutamate was added to the medium the liver was perfused at the end of the 40min. period for 2min. with 0·9% sodium chloride solution to remove the glutamate-containing perfusion medium from the organ.

Specific activity of glutamate in medium and slices. Further indications of a permeability barrier are differences in the specific activity of glutamate in the medium and in the tissue. To measure any differences the concentration of the external glutamate has to be lower than it was in the

Table 9. *Concentration of L-glutamate in perfused rat liver and in perfusion medium under various conditions*

The livers of well-fed rats were perfused for 40min. before medium and tissue were analysed. For details of the procedure see the text.

Substrates added to perfusion medium (all 10mM)	Glutamate in medium (μmoles/ml.)	Glutamate in liver (μmoles/g. wet wt.)	Ratio $\frac{\text{glutamate in liver}}{\text{glutamate in medium}}$
None	<0·1	2·99	>30
None	<0·1	2·95	>30
Glutamate	9·81	2·04	0·21
Glutamate	9·51	4·38	0·46
Oxaloacetate, L-alanine	0·42	8·54	20·6
Oxaloacetate, L-alanine	0·36	7·37	20·6

Table 10. *Specific activity of glutamate in medium and slices on incubation of rat liver and kidney-cortex slices with* [U-^{14}C]*glutamate*

Each flask contained 4ml. of saline medium including 0·5mM-[U-^{14}C]glutamate. Initially the liver contained 2·03μmoles of glutamate/g. of tissue, the kidney cortex 5·92μmoles/g. of tissue.

Tissue	Liver			Kidney		
Incubation time (min.)...	15	30	60	15	30	60
$10^{-3}\times$ Radioactivity of medium (counts/min./ml.)	190	188	181	166	169	138
$10^{-3}\times$ Radioactivity of slices (counts/min./g.)	83	118	123	358	323	334
Ratio $\frac{\text{(counts/min./g. of slices)}}{\text{(counts/min./ml. of medium)}}$	0·44	0·63	0·68	2·16	1·91	2·42
Glutamate in medium (μmole/ml.)	0·42	0·42	0·39	0·40	0·40	0·31
Glutamate in slices (μmoles/g.)	0·68	0·77	0·68	2·26	1·62	1·06
Ratio $\frac{\text{glutamate in slices}}{\text{glutamate in medium}}$	1·62	1·83	1·75	5·65	4·05	3·42
$10^{-3}\times$ Sp. activity of glutamate in medium (counts/min./μmole)	45·2	45·2	46·2	41·4	42·2	44·0
$10^{-3}\times$ Sp. activity of glutamate in slices (counts/min./μmole)	12·2	15·3	17·9	15·8	19·9	31·5
Ratio $\frac{\text{sp. activity of glutamate in slices}}{\text{sp. activity of glutamate in medium}}$	0·27	0·34	0·39	0·38	0·47	0·72

experiments so far reported so that the amounts of glutamate in the medium and in the slices are of the same order of magnitude. The results of such an experiment in which the concentration of the added [U-^{14}C]glutamate was 0·5mM, i.e. in the physiological range of blood plasma, are shown in Table 10. In the liver the ratio of the specific activity of glutamate in the tissue to that in the medium was 0·27 after 15min. and rose slowly to 0·39 within 60min. In kidney cortex the rise was more rapid and reached 0·72 after 60min. The concentration of glutamate in both liver and kidney was considerably lower than that of the freshly cut slices. In the liver it remained about one-third of the starting value throughout the incubation. In the kidney it was less than one-half the original value at 15min. and fell to about one-sixth after 60min. There was no measurable increase in the glutamate concentration of the medium. The glutamate removed from the tissue must thus have been metabolized. In these experiments the overall uptake of glutamate by the tissue was relatively slight owing to the low concentration of the added glutamate. The slow flux of glutamate through the tissue at the low external concentration may account for the fact that the specific activity of the intracellular glutamate remained below that of the medium in both liver and kidney.

Fate of added labelled glutamate in rat liver and kidney-cortex slices. When [U-^{14}C]glutamate (1mM) was added to rat liver slices, 78·9% of the radio-

Table 11. *^{14}C-labelled metabolites in the medium after incubation of rat liver and kidney-cortex slices with [U-^{14}C]glutamate*

Liver slices (100mg.) and kidney-cortex slices (50mg.) were incubated for 60min. with 4ml. of 1mM-[U-^{14}C]-glutamate and 10mM-L-lactate. The initial radioactivities were $4{\cdot}59 \times 10^5$ counts/min./ml.

Metabolite	Radioactivity (% of total) Liver	Kidney
Glutamate	78·9	66·5
α-Oxoglutarate	19·7	0·8
Glucose	1·0	22·5
Glutamine	0·3	7·1
Alanine	<0·1	3·0

activity was recovered as glutamate and 19·7% as α-oxoglutarate (Table 11). Only traces of the radioactivity appeared in glucose (1%) and glutamine (0·3%). Thus the only major reaction of glutamate that had taken place was conversion into α-oxoglutarate. Since transaminase is known to be released by the liver (see Schmidt & Schmidt, 1967) together with amino acids, it is likely that virtually all the glutamate that had reacted was removed by transaminase reactions in the medium. In the kidney, on the other hand, more glutamate was removed and 66·5% of the total radioactivity was recovered in glutamate. The remainder was recovered as glucose (22·5%), glutamine (7·1%) and alanine (3·0%). Only a trace (0·8%) appeared in α-oxoglutarate. The experiments confirm that liver slices, in contrast with kidney slices, do not readily metabolize glutamate.

Comparison of the rates of substrate removal in perfused and homogenized organs. A striking demonstration of the permeability barriers in the liver for dicarboxylic acids is provided by comparison of the rates of removal of added substrates in the perfused liver and in liver homogenates. In the homogenates the rates of removal of glutamate, α-oxoglutarate, malate and fumarate were 20–30 times, and those of succinate more than 100 times, as high as in the intact organ, whereas the rates of pyruvate removal were not very different (Table 12). Lactate was not utilized at all by the homogenate. In contrast with glutamate, glutamine showed relatively small differences. To obtain maximal rates, the homogenates were fortified with NADH and ATP, and cysteinesulphinite was added to prevent inhibitions by oxaloacetate (Singer & Kearney, 1956). The oxidizing capacities of the perfused liver and homogenates (not recorded in Table 12) were about the same. The oxygen consumption of the unsupplemented homogenates was 3·6 μmoles/min./g. and on addition of substrates it was 6·3–8·3 μmoles/min./g. The perfused liver consumed 3·5 μmoles/min./g. when lactate alone was added and over 8 μmoles/min./g. when lactate, ammonium chloride and ornithine were added (Hems *et al.* 1966).

Table 12. *Comparison of the rates of removal of added anionic substrates by the perfused rat liver and liver homogenates at 10mM-substrate and 38°*

The data for the perfused liver are taken from Table 1 of Ross *et al.* (1967). The homogenates were prepared from 1 part of liver (48hr.-starved) and 9vol. of a medium consisting of 50ml. of 0·154M-KCl, 2ml. of 0·1M-$MgCl_2$, 5ml. of 0·2M-potassium phosphate buffer, pH7·4, and 0·4ml. of 0·1M-EDTA. Warburg vessels contained 2ml. of the freshly prepared homogenate, 0·1ml. of 0·1M-ATP, 0·1ml. of 0·05M-NADH, 0·1ml. of 0·25M-substrate and saline medium or cysteinesulphinite solution to 2·5ml. Cysteinesulphinite (20mM) was added when α-oxoglutarate, succinate, malate or fumarate was the substrate to prevent the formation of inhibitory concentrations of oxaloacetate. The centre well contained 0·2ml. of 2N-NaOH and the gas space O_2. The vessels were rapidly shaken for 10 or 20min. To measure the removal of malate and fumarate the sum of malate plus fumarate was measured so that the values given represent that fraction of the substrate removed and not converted into fumarate or malate respectively. The added succinate was completely removed within 10min.; the value given is therefore a minimum rate.

Substrate added	Rate of substrate removal (μmoles/min./g.) Perfused liver	Homogenate
L-Glutamate	0·15	5·1
α-Oxoglutarate	0·19	5·6
Succinate	0·10	>12·5
L-Malate	0·23	4·5
Fumarate	0·16	4·3
Pyruvate	4·46	5·2
L-Lactate	1·95	0
L-Glutamine	1·31	3·0

In rat kidney the differences in the rates of removal between the perfused organ and homogenates were very much smaller (Table 13). Considering that the values obtained in the perfusion experiments refer to the whole kidney whereas the homogenate experiments were carried out on the cortical tissue only, the differences were negligible with malate, fumarate and pyruvate.

That glutamate can react more rapidly in liver homogenates than in liver slices was first noted by Cohen & Hayano (1946) when studying the formation of arginine from citrulline, glutamate and aspartate. Krebs, Eggleston & Hems (1948) found that the synthesis of glutamate from α-oxoglutarate and ammonium chloride in rat liver homogenates is much faster than in slices. In kidney cortex there were no such differences.

Table 13. *Comparison of the rates of removal of substrate by the perfused and homogenized rat kidney at* 10 *mM-substrate and* 38°

The data for the perfused kidney are taken from Table 3 of Nishiitsutsuji-Uwo, Ross & Krebs (1967) and recalculated for wet weight by dividing by 4·2. The homogenates were prepared from cortex only. Otherwise the procedure was as described in Table 12.

	Rate of substrate removal (μmoles/min./g.)	
Substrate added	Perfused kidney	Homogenate
L-Glutamate	2·1	6·3
Succinate	4·2	>12·5
L-Malate	3·5	4·4
Fumarate	3·3	3·7
Pyruvate	4·9	6·3
L-Lactate	1·6	6·8

DISCUSSION

The main facts on the penetration of glutamate (and other multivalent anions) into liver tissue are the following. (1) On addition of [U-^{14}C]glutamate to the external medium (the medium suspending slices or the perfusion medium) the concentration of the added substance in the tissue rose within minutes to about 50% of the external concentration and subsequently stayed near this level. (2) [U-^{14}C]Glutamate yielded $^{14}CO_2$ in the liver. As the mitochondria are the only site of this reaction glutamate carbon must have entered the mitochondria. (3) [U-^{14}C]Succinate gave results similar to those of glutamate. (4) On addition of glutamate and other multivalent anionic substrates to liver homogenates the substrates were removed by the tissue about ten times as fast as by the intact perfused rat liver. (5) Glutamate synthesized within the liver cell from precursors that rapidly penetrate (such as oxaloacetate plus pyruvate plus alanine or ammonium chloride) was largely retained by the tissue and accumulated, indicating that the permeability restrictions operated in both directions. This conclusion is further borne out by the fact that the specific activity of tissue glutamate remained lower than that of the [U-^{14}C]glutamate added to the medium. (6) Though the entry of polyanions into isolated mitochondria is somewhat restricted (Peters, 1952; Dickman & Speyer, 1954; Schneider, Striebach & Hogeboom, 1956; Amoore, 1958; Van den Bergh & Slater, 1962; Meijer & Tager, 1966; de Haan & Tager, 1966), these restrictions are readily overcome when the mitochondria are allowed to respire (Harris, van Dam & Pressman, 1967; Haslam & Krebs, 1968), or when certain additional substances (Chappell & Haarhoff, 1967) are available. (7) The hepatic 'extracellular' area, i.e. the area readily accessible to inulin, is about 25% of the tissue. (8) Anaerobically added glutamate reaches higher intracellular concentrations in the liver than aerobically.

These facts are in agreement with the assumption that the 'extracellular space' of about 25% of the liver readily communicates with the outer medium or circulating plasma and that the communication between extra- and intra-cellular area is so restricted that the intracellular concentration of added glutamate stays at about one-third of the extracellular concentration when the tissue is incubated with 5 mM-glutamate (Table 1) or 10 mM-glutamate (Table 8). This would account for an observed overall concentration of the added glutamate in the tissue of 50% of that of the medium.

Though it is possible to describe characteristic properties of the membranes that restrict or facilitate the entry of metabolites into liver compartments, the nature of the mechanism that imparts these properties to the membranes is obscure. It is one of the major characteristic properties that these membranes are permeable to large molecules such as plasma proteins as well as to low-molecular anions, cations and uncharged molecules. But while being permeable to molecules of various sizes and charges the membranes are also endowed with the capacity to regulate the rate of passage with and against concentration gradients, and in the steady state to maintain high concentration gradients. Examples of high concentration gradients are those of K^+ and of amino acids, the concentrations of which in the liver are 10–20 times as high as those of the extracellular space. With the rapid exchange between extra- and intra-cellular space detectable with isotopic K^+ (Noonan, Fenn & Haege, 1941*a*,*b*; Fenn, Noonan, Mullins & Haege, 1941–42) indicates that K^+ is constantly lost from, and recovered by, the tissue.

The outcome, then, is the conclusion that there is in the liver a major restrictive barrier at the outer cell membrane. Whether there are additional compartments within the cell, as suggested by the observation by Berl, Takagaki, Clarke & Waelsch (1962), is an open question. The different tissue pools of glutamate demonstrated by Berl *et al.* (1962) are not necessarily located within the same cell population, but may be due to the existence of different types of cells such as those of the extracellular area, the Kupffer cells, and parenchymal cells. There is no evidence that the distribution of glutamate within the organelles of the parenchymal cells is uneven in a major way. The fact that amino acids are mainly required at the site of protein synthesis, i.e. in the cytoplasm, suggests that it is this compartment where their concentra-

tion is 10–20 times that in the blood plasma (Van Slyke & Meyer, 1913; Berl *et al.* 1962; Schimassek & Gerok, 1965).

In the kidney the restrictive barrier at the outer cell membrane is very much less effective in preventing the entry of metabolites into the cells, but the fact that the concentration of many cell constituents, e.g. K^+ and amino acids, in the kidney is very different from that of the plasma or surrounding medium indicates that there is a barrier at the renal cell surfaces also.

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