Nutritional aspects of amino acid metabolism

1. A rat liver perfusion method for the study of amino acid metabolism

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1. Experiments were done to find whether the rat liver can be maintained in a satisfactory condition when perfused with oxygenated Krebs–Ringer bicarbonate buffer without added protein or red cells.

2. The condition and performance of the liver in this system were assessed from measurements made to ascertain its general condition or viability, its basal characteristics and its response to added substrates.

3. It was found that the rapid flow-rate of the medium through the livers and the efficient oxygenation of the medium ensured that enough oxygen was available for the livers to deal with large quantities of added lactate.

4. The potassium concentrations in the livers and the rates of leakage of alanine aminotransferase (*EC* 2.6.1.2) from the cells during perfusion, and the water content after perfusion showed that the livers were not grossly damaged and that they did not deteriorate measurably for up to 3 h of perfusion.

5. Liver oxygen consumption, ATP concentrations, lactate and pyruvate concentrations and ratios, and rates of urea and glucose synthesis and bile secretion, all in perfusions without added substrate, were either similar to measurements by other workers from livers perfused with media containing red cells and protein or were reasonable extrapolations from available data.

6. The rates of glucose production from lactate, and urea and glucose output from amino acids indicated that the liver responds adequately to added substrates.

7. Measurements of amino acid concentrations in perfusate indicated that the livers of rats starved for 18–20 h regulated the amino acids to characteristic levels, by overall output or uptake, except for valine, leucine and isoleucine which were continuously given out into the medium. The results suggest that in vivo there is a general flow of most of the amino acids from extrahepatic tissues to the liver during fasting, while valine, leucine and isoleucine flow from liver to extrahepatic tissues.

8. When pentobarbitone sodium (Nembutal) was used as the anaesthetic for removal of the liver from the donor rat, the rates of urea and glucose output in perfusions without added substrates were lower than when halothane (Fluothane) was used, indicating that pentobarbitone has an inhibitory effect on these measures of liver function during the subsequent perfusion.

Perfusion of the isolated rat liver with blood leads to difficulties of interpretation because of the chemical reactions which take place in the cells and plasma of the blood. These reactions cannot easily be distinguished from changes caused by the liver by conducting simple 'blank' experiments without the liver because of the influence of one of these tissues on the metabolism of the other. Other difficulties connected with the use of blood, such as its variable and relatively unknown composition, the danger of haemolysis and the possible effects of heparin on the metabolism of the liver (Coon & Willis, 1966), have been overcome to some extent by using simplified media containing protein or dextran, washed red cells and, in most instances, various

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substrates such as glucose, lactate, pyruvate or amino acids (Matschinsky, Meyer & Wieland, 1960; Mortimore, 1961; Schimassek, 1962; Schnitger, Scholz, Bücher & Lübbers, 1965; Hems, Ross, Berry & Krebs, 1966; Exton & Park, 1967); problems of interpretation would be diminished still further, particularly for studies of amino acid metabolism, if the liver could be made to function satisfactorily with a purely saline medium. Various attempts have been made, with limited success (Trowell, 1941–2; D'Silva & Neil, 1954; Dawkins, Judah & Rees, 1959; Bristow & Kerly, 1964), and this paper presents the results of a study to find whether, in fact, the liver can be maintained with oxygenated Krebs–Ringer bicarbonate buffer in as good condition as with the more complex media. Three aspects of the state of the liver in this system were studied: (1) the general condition, or viability, (2) basal characteristics, and (3) response to added substrates.

It is well known that when the liver is damaged, for instance by hypoxia, among the first measurable changes are effects on ion transport, water uptake and leakage of some intracellular enzymes (see, for example, Shoemaker & Elwyn, 1969; Rees & Shotlander, 1964; McLean, Ahmed & Judah, 1964; Magee, 1966; Popper & Schaffner, 1957; Hess, 1963; Schmidt, Schmidt, Horn & Gerlach, 1963; Bennett, 1964). The concentrations of certain cofactors and intermediates may also be expected to change early during the onset of any deterioration in condition (Scholz, Thurman, Williamson, Chance & Bücher, 1969; Bücher, Krejci, Russmann, Schnitger & Wesemann, 1964; Hohorst, Kreutz & Reim, 1961). Accordingly the condition of the liver was assessed by measuring liver and perfusate potassium concentrations, liver concentrations of adenine and pyridine nucleotides, lactate and pyruvate as well as water content and leakage of alanine aminotransferase (*EC* 2.6.1.2). These experiments were also designed to find the most acceptable criteria of the condition of the liver for routine use. As examples of the basal behaviour of the liver, the rates of glucose output, urea synthesis and bile secretion were compared with results from work with other perfusion systems and from whole animals; and the response of the liver to added lactate and added amino acids was investigated. In addition, oxygen uptake was measured, as adequate oxygen consumption is essential for a successful liver perfusion, and a lack of adequate oxygen supply is one of the most likely drawbacks to a system of this type.

Some of the amino acid results given in this paper have already been reported (Bloxam, 1966).

METHODS

The perfusion system

Apparatus. The perfusion apparatus (Fig. 1) was basically that of Miller, Bly, Watson & Bale (1951) as adapted by Fisher & Kerly (1964) but modified to make it suitable for the use of Krebs–Ringer bicarbonate buffer as perfusate instead of whole rat blood. (See Fisher & Kerly (1964) for full details of the method.) For oxygenation of the perfusate, a glass bead column (4·0 cm internal diam., containing a 16 cm depth of 8 mm diam. beads) was introduced in place of the blood film oxygenator. The humidified O_2–CO_2 (95:5) mixture was passed into the middle rather than the bottom

of the column because of the resistance of the beads, the column being open to the atmosphere at both ends (see Fig. 1). This arrangement allowed a more efficient exchange of gas with the descending perfusate. The oxygen content of the perfusate entering the liver was nearly that in fully equilibrated Krebs–Ringer bicarbonate:

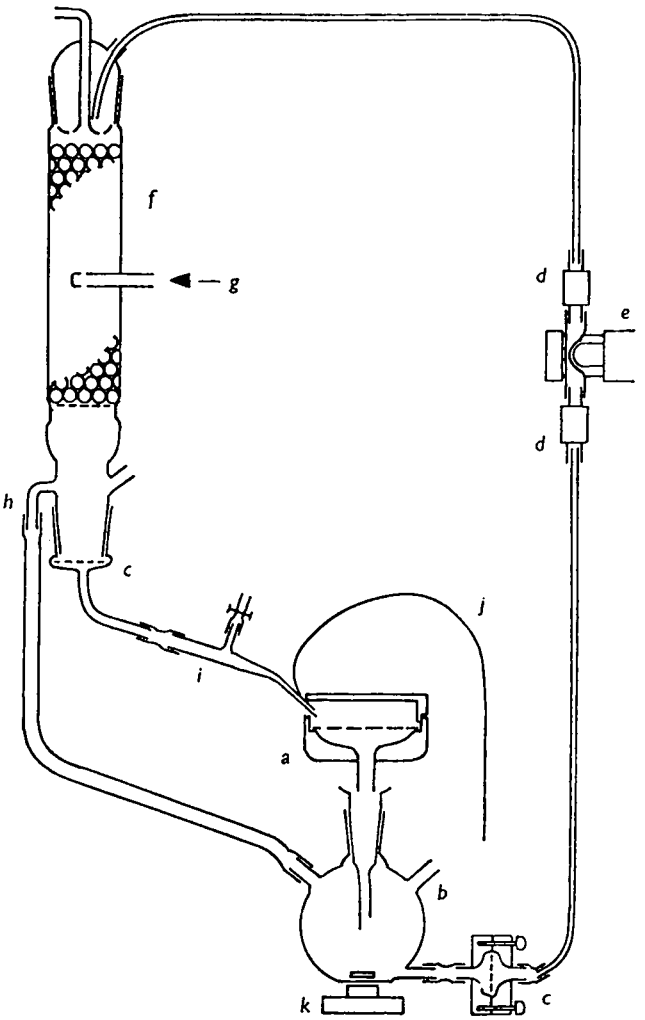

Fig. 1. Diagram of the perfusion apparatus. *a*, liver chamber; *b*, perfusion medium reservoir; *c*, filters; *d*, one-way valves; *e*, piston pump; *f*, oxygenation column; *g*, humidified O_2/CO_2 (95:5) mixture inflow; *h*, constant head overflow; *i*, portal vein cannula; *j*, bile duct cannula outlet; *k*, magnetic stirrer.

93 ± 2 (SEM) % (7 observations) after 15 min of perfusion and 94 ± 3 (7) % after 100 min. The pH was maintained between 7·4 and 7·3 even in the presence of large quantities of oxidizable substrates. The two filters contained pads of absorbent surgical gauze instead of nylon stocking and the apparatus was not silicone-treated. The pH electrodes were not inserted except when the pH of the perfusate was specifically to be

measured because of the possibility of potassium ions leaking from the calomel electrode into the perfusate and invalidating potassium determinations.

Perfusion medium. The basal perfusion medium was Krebs–Ringer bicarbonate buffer (Umbreit, Burris & Stauffer, 1964), oxygenated with a humidified O_2 (95%) and CO_2 (5%) mixture, without any other additions. The volume of medium used in each perfusion was 100 ml.

The flow-rates of perfusate through livers removed under pentobarbitone anaesthesia were $5{\cdot}8 \pm 0{\cdot}3$ ml/min.g wet weight (14 observations) after 15 min of perfusion and $6{\cdot}1 \pm 0{\cdot}3$ (15) ml/min.g after 100 min. When livers were removed under halothane anaesthesia the flow-rates were significantly greater: $9{\cdot}9 \pm 0{\cdot}5$ (11) ml/min.g after 15 min ($P < 0{\cdot}001$) and $10{\cdot}8 \pm 0{\cdot}4$ (11) ml/min.g after 100 min ($P < 0{\cdot}001$). The perfusion pressure in all instances was 13–14 cm of water, which is within the normal range of portal vein pressure in the rat (Kunkel & Eisenmenger, 1949). These values compare with 1·5–1·6 ml/min.g found when rat blood was used as the medium under essentially the same conditions, with pentobarbitone as anaesthetic (Fisher & Kerly, 1964).

When substrates were present in the medium at the start of perfusion, they were dissolved in the 0·15 M-NaCl during preparation of the medium, and the pH was adjusted to 7·4 before addition of the remaining salt solutions (Umbreit *et al.* 1964). Substrates added during perfusion were dissolved in 1·0 ml 0·15 M-NaCl, adjusted to pH 7·4 and added to the perfusate reservoir.

Operation procedure. This was as described by Fisher & Kerly (1964), except that the hepatic artery was not tied until after establishment of the oxygenated pre-perfusion, so that at no time was the liver entirely without oxygen. The total operation time was also shortened to 15–20 min by reducing the preliminary stripping and clearing of vessels to a minimum, and, where possible, by cutting adhesions to surrounding tissue during instead of before the removal of the liver from the rat.

Transfer technique. The pre-perfusate was 0·15 M-NaCl, equilibrated with 100% O_2 and maintained at 37° by pumping water at the appropriate temperature through a water jacket surrounding the pre-perfusate aspirator (see Fisher & Kerly, 1964). The use of Krebs–Ringer bicarbonate as pre-perfusate appeared to give less satisfactory results as judged by the criteria adopted in this study (see p. 415). This may have been due to the absorption by the liver of calcium ions, which probably takes place concomitantly with the loss of intracellular potassium during the removal and setting up of the liver (D'Silva & Neil, 1954) and which probably causes liver damage (Judah, Ahmed & McLean, 1964).

Anaesthetics. The anaesthetic used initially for the removal of the liver was pentobarbitone sodium (veterinary Nembutal, Abbott Laboratories) injected peritoneally (48 mg/kg body-weight). These early experiments suggested that the use of pentobarbitone as anaesthetic for removal of the liver might affect its behaviour during the subsequent perfusion. Later work was therefore carried out with a second anaesthetic, halothane (Fluothane; Imperial Chemical Industries) which was found to be more suitable (Bloxam, 1967*a*, *b*). For induction of halothane anaesthesia the rat was placed, in its cage, inside a transparent plastic anaesthetizing chamber and left for 20–30 min

to settle down. Oxygen was bubbled through the liquid halothane in a bottle immersed in a 20° water-bath, and the oxygen–halothane mixture so obtained was passed into the chamber. In early experiments with halothane, anaesthesia was maintained after induction by blowing oxygen into a beaker containing cotton-wool, moistened with halothane and held over the rat's head, the required depth of anaesthesia and adequate respiration being maintained by adjustment of oxygen flow and position of beaker. Subsequently, a modified Goldman halothane vaporizer (British Oxygen Company) was employed to deliver an oxygen–halothane mixture of adjustable composition, at a rate of 2 l/min, through a mask fitted over the head of the animal.

Animals. The liver donors were 260–280 g male albino rats, bred in this department and starved for 18–20 h. Rats for control determinations were fasted for 18–22 h and were of the same weight and type. Note that the control livers for potassium measurements were treated differently from those for the other experiments (see below).

Analysis of liver and perfusate

Oxygen consumption of the liver during perfusion was calculated from the difference in oxygen content of the perfusate entering and leaving the liver, measured by the method of Van Slyke & Neill (1924) or by oxygen electrode (model 160 Physiological Gas Analyser; Beckman Instruments; kindly made available by Dr R. Boyd, Department of Paediatrics, University College Hospital, London).

Potassium was determined by flame photometry either directly on diluted perfusate samples, correction being made for evaporation during perfusion, or on samples of fresh or dried liver, solubilized in a mixture of nitric and sulphuric acids. Samples were taken from livers after 3 h of perfusion, from livers perfused for zero time (i.e. removed from donor rats under normal operating conditions including pre-perfusion) and from control livers, rapidly perfused free from red cells *in situ* with oxygenated Krebs–Ringer bicarbonate at 37° (see p. 400).

Adenine and pyridine nucleotides were measured as follows. After perfusion for 2 h, livers were dropped into liquid nitrogen and samples of the frozen tissue were stored in liquid nitrogen until required for assay. ATP was measured by essentially the method of Lamprecht & Trautschold (1963) on neutralized perchloric acid extracts of the frozen tissue, and the pyridine nucleotides by the method of Slater, Sawyer & Sträuli (1964). Control concentrations of ATP were measured in vivo by the method of Adam (1963) in neutralized perchloric acid extracts of liver, frozen *in situ* with Wollenberger clamps (Wollenberger, Ristau & Schoffa, 1960), from animals under halothane anaesthesia.

Lactate and pyruvate concentrations of perfused livers and controls were measured in neutralized perchloric acid extracts, obtained as described in the preceding paragraph, by essentially the methods of Hohorst (1963) and Greengard (1956) respectively. For determination of concentrations in perfusate, samples of medium were mixed with 3 vol. of ice-cold 6% perchloric acid, cooled in ice, centrifuged to remove protein and neutralized with a mixture of 6 N-KOH and 0·6 N-KH_2PO_4, before assay by the same methods.

Alanine aminotransferase activity in the perfusate was determined by the method of Mohur & Cook (1957).

Liver water and perfusate total solids were measured by drying the left lobe of the livers and portions of medium respectively to constant weight at 110°.

Protein concentrations in the perfusate were assayed by the method of Lowry, Rosebrough, Farr & Randall (1951).

Urea concentrations in perfusates were measured by the method of Fawcett & Scott (1960), which involves the phenate–hypochlorite colorimetric assay of ammonia after urease treatment. Perfusate ammonia concentrations were measured in the same way but the urease step was omitted.

Perfusate glucose concentrations were determined, without prior deproteinization, by Marks's (1959) modification of the method of Huggett & Nixon (1957). Liver glycogen was measured as glucose by the same method after extraction of the tissue in 30% hot KOH, precipitation with ethanol and hydrolysis in 1 N-HCl.

Amino acid concentrations in perfusate were measured by a Technicon automatic amino acid analyser 20 h run system (Technicon Instruments Co., Amino Acid Analyser Handbook, 2nd issue). Perfusate samples, stored at −30°, were acidified with 0·05 vol. of 1 N-HCl and loaded immediately on the analyser column without further treatment. The amount of protein present was too small to interfere.

Results, except where stated otherwise, are given as mean values with their standard errors, with the number of observations in parentheses.

RESULTS

In this study, the term 'blank perfusion' means one in which nothing is added to the oxygenated Krebs–Ringer bicarbonate medium.

Oxygen uptake by the liver

The oxygen uptake by the livers during blank perfusions when halothane was used as the anaesthetic for liver removal was 2·95 ± 0·25 (5) ml/h.g wet weight after 15 min of perfusion and 2·58 ± 0·34 (5) ml/h.g after 100 min. These values are compared in Table 1 with values obtained by other workers for oxygen consumption by the rat liver in different perfusion systems. No information appears to be available for intact rats. The uptake by the livers in the system used by me removed about 40% of the oxygen present in the perfusate entering the livers (40 ± 3% after 15 min and 35 ± 6% after 100 min). The uptake, measured in one perfusion, of a liver taken from the donor rat under pentobarbitone anaesthesia was 2·30 ml/h.g wet weight after 15 min and 2·00 ml/h.g after 100 min. This amounted to removal of 35 and 30% of the available oxygen respectively.

The oxygen consumption was also measured in two perfusions in which the livers synthesized glucose at the maximal rate from added L-lactate (10 mM). Samples for oxygen estimation were taken 20 min after addition of lactate, during the time when synthesis was proceeding at the greatest rate. The uptakes were 4·55 and 3·90 ml/h.g, 52 and 47% respectively of the oxygen in the afferent perfusate being removed. The

Table 1. *Oxygen uptake by the rat liver in various preparations*

Reference	Preparation	Perfusion medium	Nutritional state of donor rats	O_2 consumption (ml/g wet wt.h) Time of observation	Single value or mean	Range
The present work	Isolated perfused liver	Krebs–Ringer bicarbonate buffer. No additions	18 h starved	After 15 min	2·95 ± 0·25	
				After 100 min	2·58 ± 0·34	
		Krebs–Ringer bicarbonate buffer + 10 mM-L-lactate	18 h starved	20 min after addition of L-lactate	4·23	
Bristow & Kerley (1964)	Isolated liver, perfused backwards	Krebs–Ringer bicarbonate buffer. No additions	Fed	After 15 min	0·82	0·81–0·84
				After 30 min	0·89	0·84–0·95
D'Silva & Neil (1954)	Isolated liver, perfused backwards	Krebs saline with haemoglobin	Fed	After 30 min	2·8*	2·4–3·2*
Hems, Ross, Berry & Krebs (1966)	Isolated perfused liver	Krebs–Ringer bicarbonate buffer with added bovine plasma albumin and aged erythrocytes	48 h starved	After 30 min	3·4†	
Schimassek (1962)	Isolated perfused liver	Saline medium with added glucose, lactate, pyruvate, bovine plasma albumin and erythrocytes		45–160 min	3·4†	
Schnitger, Scholz, Bücher & Lübbers (1965)	Isolated perfused liver. Back pressure applied to hepatic vein	Saline medium with glucose, lactate, amino acids and dextran				3·4–5·6‡
Forsander, Räihä, Salaspuro & Mäenpää (1965)	Isolated perfused liver	Diluted bovine blood	Fed	After 60 min	3·8†	
			48 h starved	After 60 min	1·7†	
Ostashever, Gray & Graff (1960)	Isolated perfused liver	Diluted rat blood with added glucose and amino acids		After 60 min	4·2§	
				After 240 min	3·1§	
Brauer (1963)	Isolated perfused liver	Whole rat blood	Fed		7·7	
Trowell (1941–2)	Estimate from survey of various sources, including liver slices				2* (approx.)	

* Calculated from Q_{O_2}, assuming a water content of 72·4% (w/w).
† Calculated from a molar uptake value.
‡ Calculated from an uptake value expressed in atmospheres pressure.
§ Assuming liver wt to be 13 g (actually, range of 12–14 g).

adequacy of the oxygen supply in the preparation is discussed on p. 412, but it may be emphasized here that the high flow-rate of medium through the livers in this preparation (see p. 396) makes available considerably more oxygen than in previous preparations with saline media (Bristow & Kerly, 1964; Dawkins *et al.* 1959; D'Silva & Neil, 1954; Trowell, 1941–2).

Assessment of the general condition of the liver

Perfusate potassium. To detect small changes in liver potassium and to obtain a continuous measure of the state of the liver throughout a perfusion, perfusate rather than liver potassium concentrations were determined. The low perfusate concentration of potassium compared with that of the liver allowed a loss of 2% from the liver to be measured without difficulty.

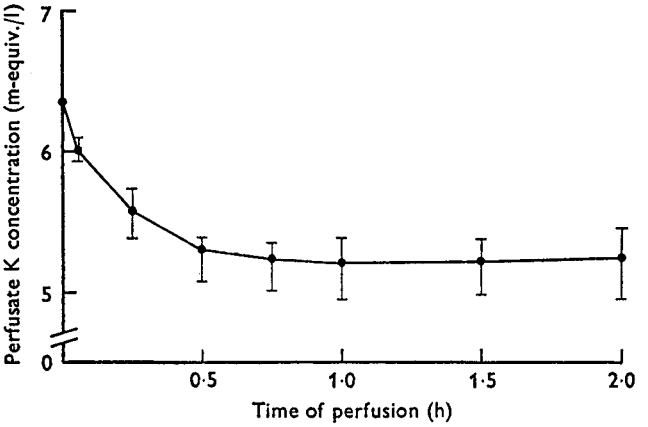

Fig. 2. Change in perfusate potassium concentration during blank perfusions of rat livers. The bars indicate the ranges over four perfusions. The livers, weighing 7·19, 7·36, 7·23 and 7·33 g, mean 7·28 g, were removed under pentobarbitone anaesthesia.

Fig. 2 shows how the perfusate potassium concentration varied during liver perfusions with pentobarbitone as anaesthetic for liver removal. During the first 20–30 min there was a rapid fall in perfusate potassium as the liver recovered the potassium it lost during its removal from the rat and insertion into the perfusion circuit. This recovery amounted to about 15% of the original liver content. Thereafter the perfusate concentration remained constant, showing that there was no subsequent leakage from the liver. These particular perfusions were run for 2 h but identical results were obtained from 3 h perfusions. Results were similar when halothane was used for the operation to remove the liver, except that the initial recovery was slightly more rapid.

'Control' livers (i.e. those rapidly perfused *in situ* to remove blood) and livers perfused for zero time (see p. 397) contained 96·6 ± 1·5 (8) and 101·2 ± 2·4 (14) μequiv. K/g wet weight respectively, the difference between these groups being not statistically significant. The potassium content of livers after 3 h of blank perfusion was 112·7 ± 2·0 (6) μequiv./g, a value which is higher than the mean control value by 16·1 μ-equiv./g ($P < 0{\cdot}001$) and higher than the value for zero time perfused livers by 11·5 μequiv./g ($P < 0{\cdot}01$). This is in general agreement with the mean potassium

recovery of 15·8 μequiv./g by the livers in the perfusions shown in Fig. 2, calculated from the fall in perfusate potassium (see also values in Table 2). The liver potassium concentrations also indicate that most of the potassium loss took place during laparotomy and cannulation rather than during liver removal and pre-perfusion.

The highest published values for the potassium content of rat liver, found in recent literature, are 106·0 ± 0·68 (93) μequiv./g wet weight (Weiss, 1966) and 107·8 ± 3·3 (8) μequiv./g (Herken, Senet & Zemisch, 1966), and most values range from 95 to 100 μequiv./g (see, for instance, Spector, 1956). Comparison of these results with the values obtained in this work suggests that levels in vivo are nearer those in the livers after perfusion than in the control livers. The results also suggest (but do not prove) that the recovery of potassium by the liver during the first few minutes of perfusion approached 100%. Thus the quantity of potassium taken up is a measure of that lost during the operation and transfer and is consequently an indication of the degree of damage sustained. In addition, the rate of regain of potassium is an indication of the rate of recovery of the liver.

Liver adenine and pyridine nucleotides. In order to find if nucleotide determinations might provide acceptable indications of the condition of the liver during perfusion, concentrations were measured in livers after normal perfusions and also after some in which the livers were unsatisfactory, but not grossly damaged, owing to prolongation of time of cannulation up to 1 min or owing to an increased duration of transfer and establishment of the perfusion circulation. Damage was indicated by increased uptake of potassium by the livers, and those whose uptake was greater than 20 μequiv./g were arbitrarily called 'unsatisfactory'. In no instance was there any significant potassium loss during perfusion.

Table 2 shows total liver concentrations of ATP, and of the oxidized and reduced forms of NAD^+ and $NADP^+$, after 2 h of normal and unsatisfactory blank perfusions, together with the initial potassium recoveries. The ATP concentrations were significantly reduced in the unsatisfactory perfusions, but there was no significant difference in concentration, sum or ratio of any of the pyridine nucleotides (see Table 4). This is consistent with the finding of Hohorst *et al.* (1961) that anoxia for periods between 10 and 60 s caused no change in total cell NADH to NAD^+ ratio in vivo, although the lactate to pyruvate ratio was increased. The ATP concentration of 2·17 ± 0·09 μmol/g wet weight after 2 h of perfusion is similar to values of 2·05 ± 0·15 μmol/g after 85 min found by Hems *et al.* (1966) using livers of rats starved for 48 h perfused with a medium based on Krebs–Ringer bicarbonate containing protein and red cells, 2·27 μmol/g after 30 min by Schimassek (1963) with livers of fed rats perfused with a similar medium based on Tyrodes solution and 1·96 ± 0·17 μmol/g after 30 min by Söling, Willms, Friedrichs & Kleineke (1968) with livers of fed rats perfused with Tyrodes solution containing protein, red cells, glucose, lactate and pyruvate. All these values are somewhat below those obtained for rat liver in vivo. For example, the ATP concentration in livers of control rats starved for 18–24 h and under halothane anaesthesia was 3·08 ± 0·09 μmol/g (13), similar to values found by others in fed rats (Bartels & Hohorst, 1963; Bücher *et al.* 1964; Hohorst, Kreutz & Bücher, 1959).

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Table 2. *Potassium recovery from perfused rat livers removed under halothane anaesthesia, and their adenine and pyridine nucleotide* content measured after 2 h of blank perfusion*

(Mean values with their standard errors)

	No. of perfusions	Initial potassium recovery (see p. 401) (μequiv./g)	ATP (μmol/g)	NADH (μmol/g)	NAD^+ (μmol/g)	NADH + NAD^+ (μmol/g)	$\frac{NAD}{NADH}$	NADPH (μmol/g)	$NADP^+$ (μmol/g)	NADPH + $NADP^+$ (μmol/g)	$\frac{NADPH}{NADP^+}$
Normal perfusions	4	17·4 ±0·8	2·17 ±0·09	0·043 ±0·005	0·458 ±0·028	0·501 ±0·028	0·096 ±0·013	0·131 ±0·007	0·243 ±0·033	0·374 ±0·032	0·59 ±0·10
Unsatisfactory perfusions (see p. 401)	4	24·9 ±1·9	1·53 ±0·10	0·036 ±0·005	0·522 ±0·045	0·558 ±0·045	0·070 ±0·014	0·107 ±0·024	0·266 ±0·076	0·373 ±0·096	0·45 ±0·10

See Table 4 for a statistical comparison of the two sets of results.

* Kindly measured by Dr T. F. Slater, Department of Chemical Pathology, University College Hospital Medical School, London.

The NADH to NAD^+ ratio of $0{\cdot}096 \pm 0{\cdot}013$ found here is similar to values of 0·077 and 0·060 in livers of rats starved overnight perfused with a medium containing protein and initially 10 mM-L-alanine after 35 and 60 min, found by Williamson, Kreisberg & Felts (1966). These workers also obtained values of 2·85 and 2·77 for the ratio NADPH to $NADP^+$ after similar times, compared with that of $0{\cdot}59 \pm 0{\cdot}10$ found here. These differences may be attributed to the different substrate conditions. No values could be found in the literature for concentrations in vivo of the pyridine nucleotides in the livers of rats starved overnight.

Table 3. *Lactate and pyruvate, after 120 min of blank perfusion, in rat livers removed under halothane anaesthesia both for perfusion and for control experiments*

(Mean values with their standard errors)

	No. of observations	Lactate (μmol/g wet wt)	Pyruvate (μmol/g wet wt)	$\frac{\text{Lactate}}{\text{Pyruvate}}$
Normal perfusions	4	$0{\cdot}69 \pm 0{\cdot}07$	$0{\cdot}072 \pm 0{\cdot}007$	$9{\cdot}6 \pm 0{\cdot}7$
Unsatisfactory perfusions	4	$0{\cdot}54 \pm 0{\cdot}22$	$0{\cdot}035 \pm 0{\cdot}016$	$16{\cdot}9 \pm 1{\cdot}4$
In vivo controls	13	$0{\cdot}38 \pm 0{\cdot}03$	$0{\cdot}027 \pm 0{\cdot}005$	$16{\cdot}8 \pm 1{\cdot}7$

See Table 4 for a statistical comparison of the results.

Table 4. *Comparison of results given in Tables 2 and 3*

	t test probability value		
Measurement	Normal perfusions *v.* unsatisfactory perfusions	Normal perfusions *v.* in vivo controls	Unsatisfactory perfusions *v.* in vivo controls
Potassium recovery	0·01	—	—
ATP	< 0·01	< 0·001	< 0·001
NADH	NS	—	—
NAD^+	NS	—	—
NADH + NAD^+	NS	—	—
NADH:NAD^+	NS	—	—
NADPH	NS	—	—
$NADP^+$	NS	—	—
NADPH + $NADP^+$	NS	—	—
NADPH:$NADP^+$	NS	—	—
Lactate	NS	< 0·01	NS
Pyruvate	NS	< 0·001	NS
Lactate:pyruvate	< 0·01	< 0·01	NS

NS, $P > 0{\cdot}10$.

Lactate and pyruvate in liver and perfusate. Lactate and pyruvate were measured in livers after blank perfusions, both normal and 'unsatisfactory' (see p. 401), in control livers from rats starved for 18–22 h and in the perfusate after 20 min and 2 h of perfusion (Table 3). Both lactate and pyruvate concentrations in livers after 2 h of perfusion were somewhat higher than in the control livers and also higher than in the unsatisfactory livers, while the lactate to pyruvate ratios were lower than in both of the other groups (see Table 4 for statistical comparisons). Williamson *et al.* (1966)

found a lactate to pyruvate ratio of 10·9 in livers of rats starved for 18–24 h, perfused for 35 min, and 8·1 when perfused for 60 min with a saline medium containing bovine serum albumin and initially 10 mM-L-alanine, but they do not state the concentrations of these intermediates. Ross, Hems & Krebs (1967) report lactate and pyruvate concentrations of $0·32 \pm 0·08$ (4) and $0·07 \pm 0·01$ (4) μmol/g wet weight respectively and a ratio of $4·6 \pm 1·2$ (4) in livers of female rats starved for 48 h, perfused for 1·4 h with Krebs–Ringer bicarbonate containing protein and red cells, compared with in vivo control values of $0·92 \pm 0·20$ (7) and $0·07 \pm 0·01$ (7) μmol/g of lactate and pyruvate, ratio $13·7 \pm 3·6$; while Krebs (1967) found in vivo values for male rats starved for 48 h of $0·78 \pm 0·40$ (23) and $0·047 \pm 0·024$ (23) μmol/g of lactate and pyruvate – ratio 17. Söling *et al.* (1968) found lactate and pyruvate concentrations of $1·62 \pm 0·19$ and $0·25 \pm 0·05$ μmol/g in livers of fed rats, perfused for 30 min with a medium initially containing protein, red cells, glucose, lactate and pyruvate, and a ratio of $6·6 \pm 1·5$ compared with the accepted in vivo ratio for fed rats of between 9·5 and 12·5 (Bartels & Hohorst, 1963; Bücher *et al.* 1964; Hohorst *et al.* 1959; Krebs, 1967).*

The work described now thus confirms that the lactate to pyruvate ratio rises when a perfusion becomes unsatisfactory and, in agreement with other workers, it also shows that the lactate–pyruvate couple under the perfusion conditions is in a more oxidized state than in vivo. This is perhaps understandable, remembering that the liver in the experiments reported here was supplied with no exogenous substrate. For instance, lactate dehydrogenase is not dealing with a continuous inflow of lactate as it is in vivo, while glyceraldehydephosphate dehydrogenase is working in the overall direction of glucose synthesis; factors which alone must affect the redox state of cytoplasmic NAD^+. The redox state of NAD^+ in the mitochondrial compartment of the perfused liver in the absence of added substrate has also been found to be more oxidized than in vivo (Krebs, Wallace, Hems & Freedland, 1969).

The perfusate concentrations of lactate in the perfusions shown in Table 3, both normal and unsatisfactory, and at both 20 min and 2 h of perfusion, were below 1 μmol/100 ml, and those of pyruvate were likewise below 0·2 μmol/100 ml, or in both instances below the limits of measurement by the methods used. Thus there was no measurable accumulation of lactate in the medium. Moreover, these values compared with tissue levels show that the liver is capable of maintaining considerable concentration gradients of both these substances at the cell membrane.

Alanine aminotransferase activity. Perfusate alanine aminotransferase activity, measured in two perfusions (these determinations were kindly carried out by Dr H. M. B. Buckell, Department of Neurosurgery, St George's Hospital Medical School, London), increased linearly at a rate of 0·037 units/g wet weight.h in one perfusion and 0·057 units/g.h in the other (1 unit of enzyme converts 1 μmol of α-amino acid

* Since the preparation of this manuscript the following results have been reported for a rat liver perfusion method in which livers of 200–250 g rats starved overnight were perfused with oxygenated Krebs–Ringer bicarbonate solution without additions. After 45 min of perfusion the liver lactate and pyruvate concentrations were $0·444 \pm 0·084$ (10) μmol/g wet weight and $0·024 \pm 0·003$ (12) μmol/g respectively, ratio 26 ± 7 (11), and the ATP concentration was $1·034 \pm 0·114$ (12) μmol/g (Toews, Lowy & Ruderman, 1970).

into 1 μmol of α-oxo acid in 1 min at 37°). The normal range of serum alanine aminotransferase activity in rats of the same stock as those used in the perfusion experiments was: for fed rats, $2{\cdot}57 \pm 0{\cdot}54$ (SD) units/100 ml (range 1·88–3·47 units, $n = 8$); for starved rats, $1{\cdot}70 \pm 0{\cdot}52$ (0·92–2·52, $n = 11$) units (J. E. L. Spruyt, personal communication).

Liver water content. The results in Table 5 show that there was a small but significant increase in the water content of livers perfused for 3 h with a blank medium of the order of 1% of the wet weight. An increase in water content after perfusion when expressed per liver weight (wet or dry) could be caused either by an uptake of water

Table 5. *Water content and wet and dry weights after perfusion of rat livers removed under pentobarbitone anaesthesia*

(Mean values with their standard errors)

	No. of observations	Wet wt (g)	Dry wt (g)	Water content: % of wet wt	Water content: Water:solid ratio
3 h perfusions	15	$7{\cdot}65 \pm 0{\cdot}14$	$2{\cdot}01 \pm 0{\cdot}03$	$73{\cdot}5 \pm 0{\cdot}2$	$2{\cdot}77 \pm 0{\cdot}032$
Unperfused controls	14	$7{\cdot}97 \pm 0{\cdot}18$	$2{\cdot}21 \pm 0{\cdot}04$	$72{\cdot}1 \pm 0{\cdot}4$	$2{\cdot}55 \pm 0{\cdot}065$
P value of t test		NS	< 0·001	< 0·01	0·01

NS, $P > 0{\cdot}10$.

(oedema) or by an output of solutes which would decrease the dry weight. In fact the dry weights of the perfused livers were slightly, but again significantly, lower than those of the controls (non-perfused livers). This, taken with the fact that no measureable increase in water is indicated by the values for wet weights, suggests (but does not prove) that the increase in water content is due to the output of liver solutes rather than a gain of water. The loss of solutes was shown by direct measurement of the dry matter of the perfusate (by evaporation to dryness at 110°), and the decrease in liver dry weight was more than enough to account for the increase in water content per unit wet or dry weight. It is not surprising that the liver should lose weight under the conditions used because the perfusate initially contained no substrates, and the liver synthesized large amounts of products which appeared in the perfusate. In fact at least 60% of this loss from the perfused liver could be accounted for by the measured output of glucose, urea, amino acids and protein alone, and it would be interesting to find how much of the remaining 40% consisted of lipid material, solutes in the bile and expired CO_2. A loss in weight and gain in percentage water content of the liver during starvation has also been found in the intact rat (Peters & Boyd, 1966).

Basal behaviour of the liver

All the results given in this and the next section are from perfusions in which the initial uptake of potassium by the liver was less than 20 μ-equiv./g wet weight, and in which there was no subsequent measurable loss of potassium. Also, the appearance of the livers was satisfactory, and the flow-rate of the medium, liver water content and bile flow-rate were all within the limits recorded for normal perfusions.

Urea production and perfusate ammonia levels. Urea production by the liver was

linear throughout perfusion for 3 h (see results for blank perfusions in Fig. 7). Table 6 shows that when pentobarbitone was the anaesthetic for removal of the liver from the donor rat the rate was $8{\cdot}17 \pm 0{\cdot}43$ (6) μmol/g wet weight.h, and when halothane was used the rate was $10{\cdot}61 \pm 0{\cdot}49$ (9) μmol/g.h ($P < 0{\cdot}01$). This suggests that when pentobarbitone is used for the operation for liver removal it has an inhibitory effect on urea output during the subsequent perfusion, and this is supported by further work (Bloxam, 1967*a*) in which the anaesthetics were added to the perfusate during perfusion. Pentobarbitone strongly inhibits urea output while halothane at high levels has a much smaller effect.

Table 6. *Rates of urea and glucose production by rat livers during blank perfusions: effect of anaesthetics*

(Mean values with their standard errors; no. of observations in parentheses)

Anaesthetic used for removal of liver	Urea production (μmol/g wet wt.h)	Glucose production (μmol/g wet wt.h)
Pentobarbitone	$8{\cdot}17 \pm 0{\cdot}43$ (6)	$4{\cdot}76 \pm 0{\cdot}31$ (6)
Halothane	$10{\cdot}61 \pm 0{\cdot}49$ (9)	$5{\cdot}87 \pm 0{\cdot}23$ (8)
P value of t test	$< 0{\cdot}01$	$< 0{\cdot}02$

The rate of urea output from livers removed under halothane anaesthesia, and perfused without added substrate, was $95{\cdot}7 \pm 0{\cdot}43$ μmol/h when expressed per whole liver. This compares with a range of 67–186 μmol/liver.h from rat livers perfused with rat blood, with or without various additions, by Burton, St George & Ishida (1960) using livers from rats starved for 18–20 h. A range of 7–18 μmol/g wet weight.h has been found by Miller, Burke & Haft (1955) from livers of rats starved for 18–20 h, perfused with blood, and $5{\cdot}4 \pm 0{\cdot}6$ (4) μmol/g.h by Hems *et al.* (1966) from livers of rats starved for 48 h, perfused with a saline medium with added red cells and protein.

Ammonia concentrations in the perfusate remained below 15 μmol/100 ml with both anaesthetics.

Glucose production and glycogen levels. Glucose is given out by the liver at a virtually constant rate throughout 3 h of perfusion (see results of blank perfusions in Fig. 7). Table 6 shows that, as with urea synthesis, pentobarbitone probably causes some inhibition of glucose output when used as the anaesthetic for liver removal. This, again, has been supported by subsequent work (Bloxam, 1967*a*). The rates in Table 6 compare with 12 μmol/g.h found by Teufel, Menahan, Shipp, Böning & Wieland (1967) for livers of rats starved for 18–20 h, perfused with a saline medium containing red cells, protein and glucose, an average of 14 ± 1 (12) μmol/g over the 1st hour of perfusion (falling off with time) by Exton & Park (1967) with livers from rats starved for 18–22 h, perfused with Krebs–Ringer bicarbonate buffer containing protein and red cells, and an average of $8{\cdot}4 \pm 1{\cdot}7$ μmol/g.h between 40 and 85 min (output decreasing with time) by Hems *et al.* (1966) with livers from rats starved for 48 h, perfused with a similar medium.

The mean glycogen concentration of four livers after removal from the rat under pentobarbitone anaesthesia and pre-perfusion with 0·15 M-NaCl solution (i.e. equi-

valent to livers perfused for zero time, see p. 396) was 140 ± 11 μg as glucose/g wet weight, and of four livers perfused for 3 h without additions to the perfusate and pentobarbitone as anaesthetic was 200 ± 11 μg as glucose/g wet weight. The glycogen content of livers of ten rats from the same stock as those used in the present work, and starved for 18–24 h was 3200 ± 500 μg/g (Fisher & Kerly, 1964). Thus approximately 95% of the liver glycogen is lost during the operation and transfer, but there is apparently neither gain nor further loss during the blank perfusion. As glucose output is linear from zero time of perfusion, this glycogen loss is presumably completed during the pre-perfusion period of transfer and the glucose produced discarded with the effusate.

In addition, since the glycogen concentrations in the preparation used in this work are very low, glucose appearing in the perfusion medium must come from immediate glucose synthesis.

Bile production. Bile production by the liver during perfusion after removal under pentobarbitone anaesthesia was $0{\cdot}41 \pm 0{\cdot}05$ (13) ml/liver.h and after halothane anaesthesia was $0{\cdot}51 \pm 0{\cdot}05$ (6) ml/liver.h (difference not statistically significant) in the earlier perfusions, but in later perfusions in which the halothane vaporizer was used for anaesthesia (see p. 397) there was evidence that bile secretion in the subsequent perfusion was slower when anaesthesia was deeper. This does not appear to be a direct inhibition by halothane because, when added to the perfusate at high concentration during perfusion, halothane had little effect on bile production (Bloxam, 1967*a*).

The rates of bile production were very similar to those obtained by other workers with livers perfused with blood or media containing protein and red cells (Brauer, Pessotti & Pizzolato, 1951; Burton *et al.* 1960; Fisher & Kerly, 1964; Gordon, 1963; Hems *et al.* 1966; Miller, Burke & Haft, 1956; Morris, 1960; Ostashever, Gray & Graff, 1960) and to those found in the intact anaesthetized (Fisher & Kerly, 1964; Krayer, 1928; Telkkä & Kuusisto, 1962) and non-anaesthetized (Friedman, Byers & Michaelis, 1950) rat, but less than that of 1·04 ml/animal.h obtained by Brauer *et al.* (1951) in 250–310 g male non-anaesthetized rats.

Response of the liver to added lactate

Glucose output. Fig. 3 shows the effect of lactate (added after 1 h to give a perfusate concentration of 10 mM) on glucose production by the liver in two perfusions. The maximum (linear) rates after the addition were 46·5 and 46·8 μmol/g wet weight.h from livers of 7·6 and 9·8 g respectively. These rates, from livers of rats starved for 18–20 h, are somewhat greater than the average of 36 μmol/g.h obtained by Williamson, Browning & Scholz (1969) and of 40 μmol/g.h by Struck, Ashmore & Wieland (1965) for livers of rats starved for 24 h, perfused with saline media containing protein without and with red cells, respectively, and rather less than rates of about 60 μmol/g.h obtained by Exton & Park (1967) and Menahan, Ross & Wieland (1968) with livers from rats starved overnight, perfused with similar media containing red cells. Ross, Hems, Freedland & Krebs (1967) report rates of $27{\cdot}6 \pm 5{\cdot}4$ (4) μmol/g.h from livers of fed rats and $60{\cdot}6 \pm 3{\cdot}6$ (5) μmol/g.h from livers of rats starved for 48 h, perfused

with Krebs–Ringer bicarbonate containing protein and red cells, both of these two groups of rats having been treated with phlorrhizin and glucagon to deplete the livers of glycogen before perfusion. These results are discussed later. At 2 h after addition of the lactate, the liver concentrations of both lactate and pyruvate and their ratios had already returned to within the normal range. The uptake of lactate by this time amounted to 99·2 and 99·5% of the amount added.

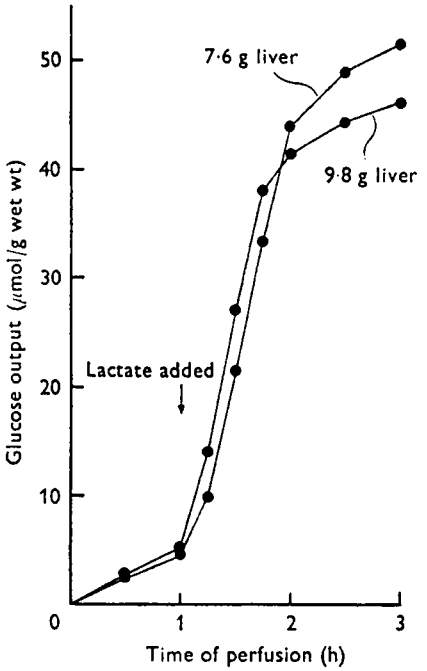

Fig. 3. Glucose production from L-lactate (10 mM) by rat livers during perfusion. The livers were removed under halothane anaesthesia. The potassium recovery of the 7·6 g liver was 18·9 μequiv./g and of the 9·8 g liver it was 10·7 μequiv./g.

Urea production. In both perfusions the urea output decreased by about 30% after the addition of lactate (10 mM) to the perfusate (Fig. 4). The decrease was not due to any limitation of oxygen availability because the perfusate after passage through the liver contained at least twice the O_2 concentration in contact with the cells in vivo (see p. 413). Moreover, there was no efflux of potassium, but, in fact, a small uptake (Fig. 4); the lactate concentrations and lactate to pyruvate ratios 2 h after addition of lactate had returned almost to the values for blank perfusions (see p. 403), and ATP levels had returned to 2·08 and 2·26 μmol/g. These findings suggest that there had been no limitation of energy supply. Thus, the reduced urea output may have resulted from reduced utilization of amino acids in the presence of a high concentration of exogenous lactate.

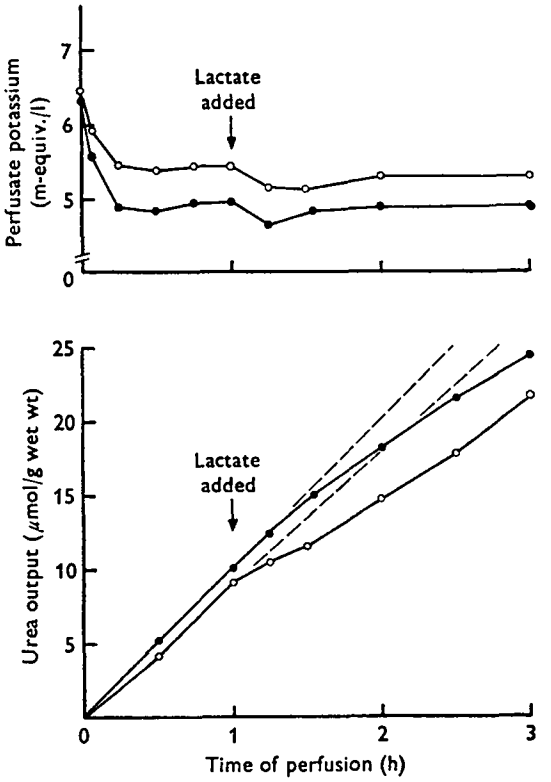

Fig. 4. Effect of added lactate (10 mM) on perfusate potassium concentration and on urea output by the perfused liver. Broken lines in the latter are extrapolations of initial urea outputs. The livers were removed under halothane anaesthesia. ●—●, 7·6 g liver; ○—○, 9·8 g liver.

Response of the liver to added amino acids

Changes in perfusate amino acid concentrations. Fig. 5 shows the changes in amino acid concentrations in the perfusates during four experiments, two of which were blank perfusions and in two of which all the protein amino acids, together with taurine, citrulline and ornithine were present in the perfusate at the start of the perfusions, at concentrations approximately equal to or somewhat above those normally found in the plasma of starved rats.

During the blank perfusions, all the amino acids normally found in blood appeared in the perfusate, each, except valine, leucine and isoleucine, at a concentration tending towards an equilibrium level. Once established, the equilibrium concentration was maintained for the remainder of the perfusions. The branched-chain amino acids, valine, leucine and isoleucine, were given out from the livers at more or less constant rates until the perfusions were stopped. These perfusions were run for 2 h, but closely similar results have since been obtained from 3 h perfusions, with both pentobarbitone and halothane as the anaesthetic for liver removal, showing that, except for the branched-chain group, each amino acid has a characteristic perfusate level in these circumstances. Those of glycine and lysine are relatively high, and those of arginine, aspartate, tryptophan and alanine are particularly low.

During the other two perfusions all the added amino acids, again with the exception of valine, leucine and isoleucine, were taken up by the liver until approximately the same perfusate equilibrium concentrations were reached, and these concentrations were maintained during the remainder of each perfusion. In some instances the final concentrations tended towards values somewhat below those of the blank perfusions and this was particularly marked for tyrosine, glycine and lysine. In contrast to the others, valine, leucine and isoleucine were not taken up by the liver, although their

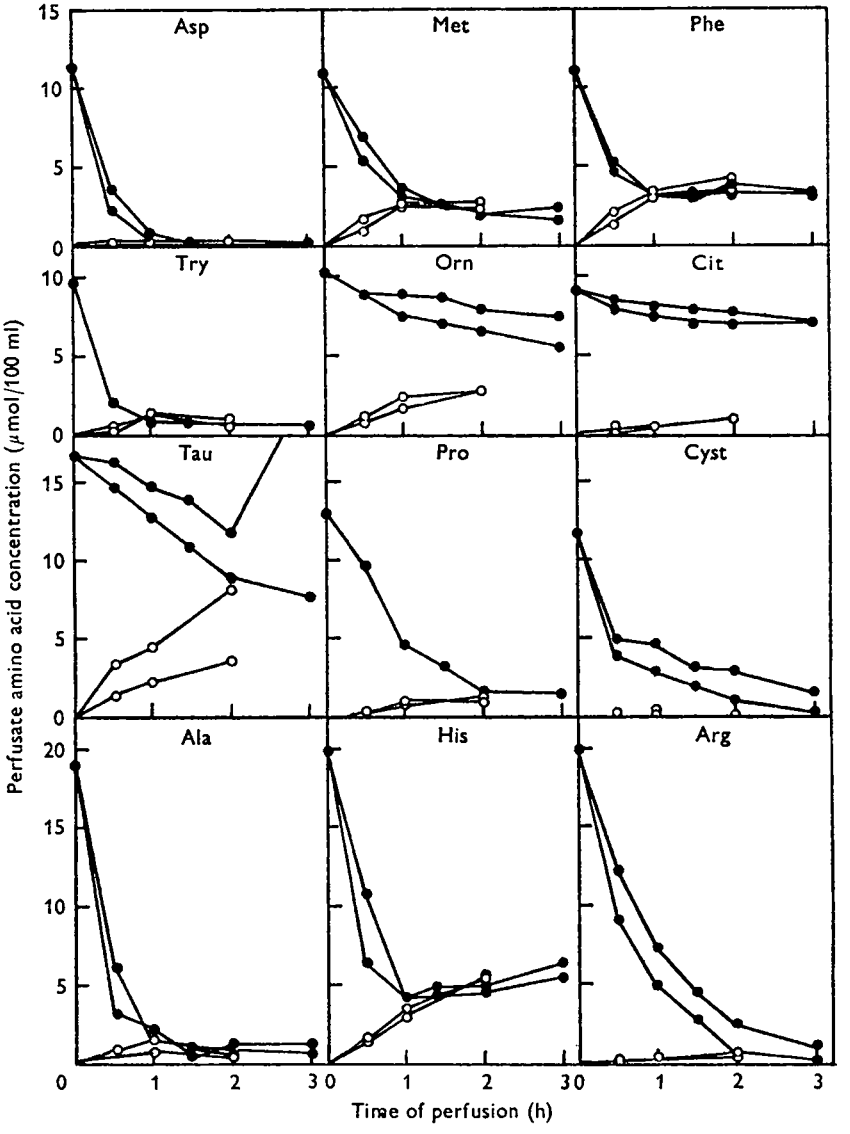

Fig. 5. Changes in perfusate amino acid concentrations during rat liver perfusions. The figure shows the perfusate concentrations in two blank perfusions (○—○), and in two with a mixture of amino acids added at zero time (●—●). For proline and tryptophan the results of one perfusion only are given because of inadequate determination of these acids in some of the samples. The livers were removed under pentobarbitone anaesthesia.

initial concentrations were high. After an initial lag period, also noticeable in the blank perfusions, and probably representing the period of recovery of the liver after its removal from the rat and transfer to the perfusion circuit, they were transported out of the liver as fast as in the blank perfusions. Again, further perfusions with different starting concentrations of amino acids have shown essentially the same results.

In a confirmatory perfusion to test that a liver could adjust the perfusate concentration of an amino acid in both directions, 10 μmol of glycine were added to the medium after 90 min of a blank perfusion. Fig. 6 shows that after the addition the

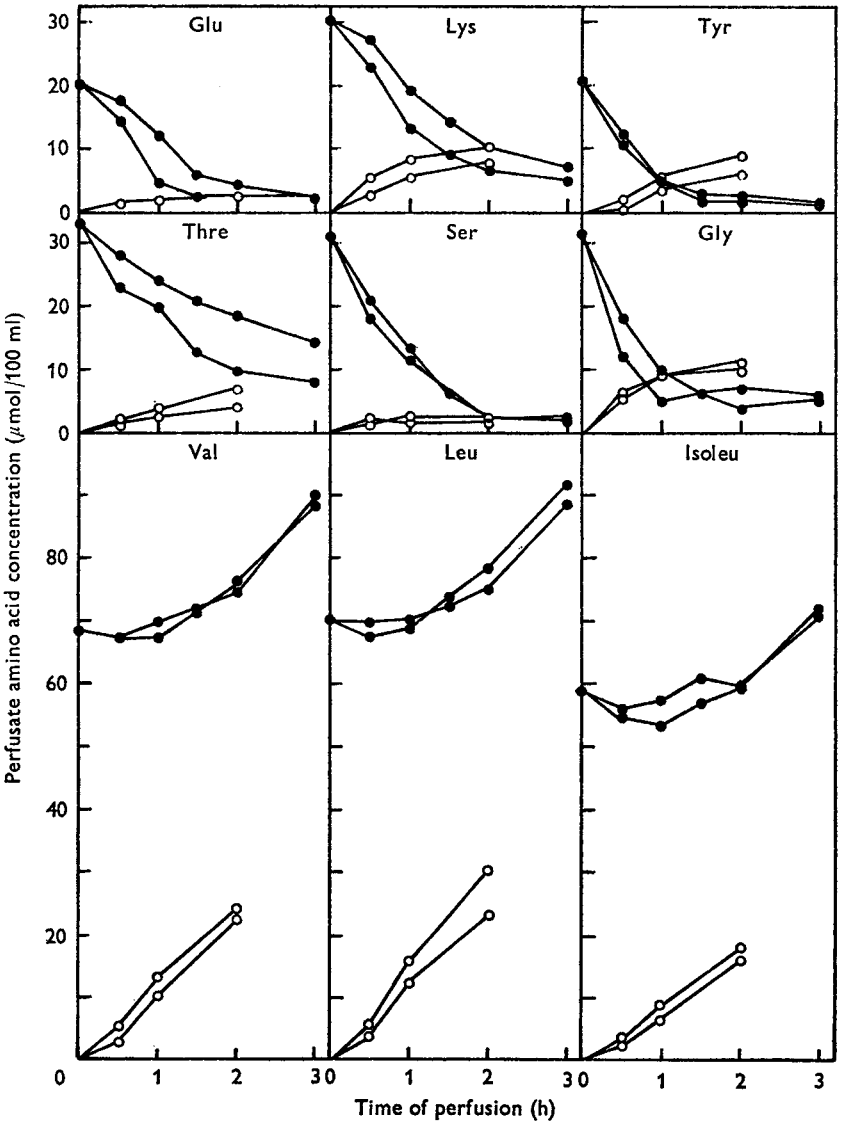

Fig. 5 (continued). For legend see opposite page.

output of glycine reverted to an uptake so that the equilibrium concentration tended to be re-established.

Urea and glucose production. The effect of an added amino acid mixture on urea and glucose output of the liver is shown in Fig. 7. In blank perfusions both urea and glucose were given out at virtually linear rates (Table 6). When a mixture of all the amino acids was added to the medium (see Fig. 5 for their initial concentrations) there was an immediate stimulation of both urea and glucose output which fell off with time, generally in parallel with the uptake of amino acids, until after 60–90 min the basal rates had been re-established.

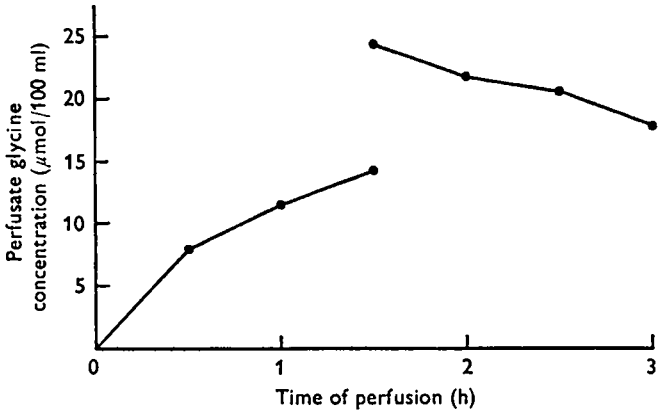

Fig. 6. Two-way adjustment of perfusate glycine concentration by the rat liver in a single perfusion. 10·0 μmol/glycine were added after 1·5 h of a blank perfusion. The liver was removed under pentobarbitone anaesthesia.

The average amount of urea produced in the two perfusions in excess of the average of the blank perfusions was 100 μmol. Since an average of 254 μatoms of α-amino nitrogen (equivalent to 127 μmol of urea) had been taken up by the livers, this represents an approximately 80% conversion into urea.

DISCUSSION

Oxygen consumption

The difficulty experienced by previous workers in obtaining suitable preparations with saline media may have been partly due to lack of adequate oxygenation. In this system more oxygen has been made available to the liver by the faster flow-rate of the Miller perfusion method compared with other systems, the improved oxygenation of the perfusate and the absence of an anoxic period during the operation and transfer procedures (see p. 396). During blank perfusions the liver removed about one-third of the oxygen present in the medium entering the livers (40 ± 3% after 15 min and 35 ± 6% after 100 min of perfusion), the oxygen remaining after passage through the livers being 0·84 ± 0·04 (5) ml O_2/100 ml perfusate after 15 min and 0·94 ± 0·05 (5) ml O_2/100 ml after 100 min (P_{O_2}: 266 ± 14 mm Hg and 300 ± 16 mm Hg

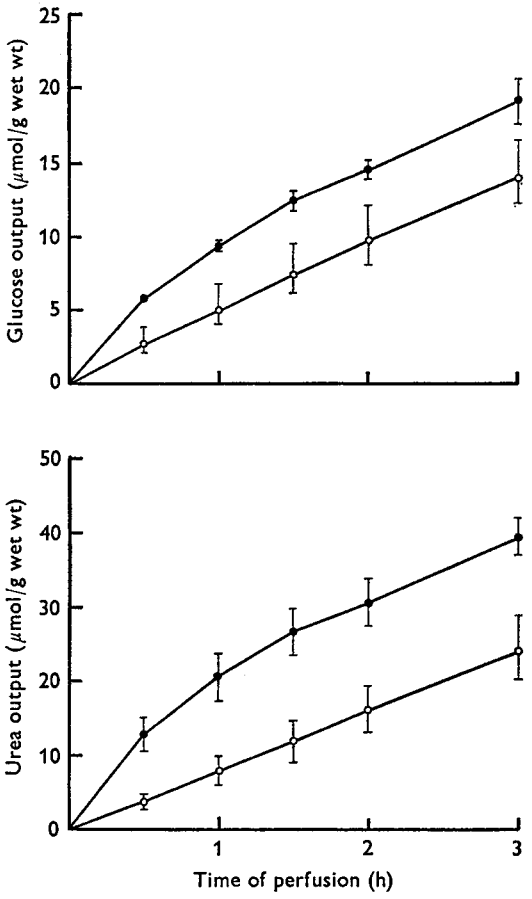

Fig. 7. Output of urea and glucose from rat livers after addition of an amino acid mixture to the medium (see Fig. 5 for their initial concentrations). The closed circles (●—●) represent the average of two perfusions; the open circles (○—○) represent the average of six blank perfusions for comparison. The bars indicate the ranges. The livers were removed under pentobarbitone anaesthesia.

respectively). During the time when the livers were synthesizing glucose maximally from lactate the livers took up half of the oxygen initially present (47 and 53% in the two perfusions), 0·60 and 0·54 ml O_2/100 ml of medium (190 and 172 mm Hg, P_{O_2}) remaining in the emergent perfusate. These concentrations are still probably two to four times those in the plasma which comes into contact with the parenchymal cell in vivo, judging from values for plasma oxygen concentrations in man (Albritton, 1952), 0·258 ml O_2/100 ml of plasma (P_{O_2}: 94 mm Hg) in arterial blood, and 0·110 ml O_2/100 ml (40 mm Hg) in venous blood (no values could be found for the rat). These measurements show that the liver is not hypoxic, even when metabolizing large quantities of lactate.

It should be mentioned, however, that this conclusion is based on the assumption that oxygen taken up by the parenchymal cells of the liver in vivo is drawn from that physically dissolved in the plasma in the perisinusoidal space. Brauer and his co-

workers have found that, when a liver is perfused with blood, the degree of oxygen saturation of the red cells and its haematocrit influence the rate of oxygen uptake (Brauer, Leong & Holloway, 1961; Brauer, Leong, Holloway & Krebs, 1963). They suggest that when the oxygen saturation of the cells or the perfusate haematocrit is lowered below normal the respiration of the liver is depressed, eventually to such an extent that the condition of the liver may be impaired. They interpret their results as possibly indicating that oxygen is transferred directly from erythrocyte membrane to parenchymal cell membrane (Brauer *et al.* 1963). On the other hand, in the present work with livers perfused with a medium containing no red cells there is no evidence that the availability of oxygen is limiting to the functioning of the liver. Perhaps the oxygen is more freely available when in physical solution than when bound in the red cells in the sinusoids.

It is well known that the rate of oxygen uptake by the liver depends on the concentration of substrates present (see, for example, Brauer, 1963; Brauer *et al.* 1963; Hems *et al.* 1966; Krebs, 1950), and the results in Table 1 seem to be more or less consistent with this. It is significant, too, that the rate of oxygen consumption of perfused livers from rats fasted for 48 h is reduced to half the value for fed rats (Forsander, Räihä, Salsapuro & Mäenpää, 1965). A similar, but even more marked lowering in respiration of the liver after fasting has been found in intact cats (Barcroft & Shore, 1913). The decrease in oxygen consumption with time during perfusion is similarly probably due to the reduction in availability of intracellular substrates. In the light of these results the oxygen uptake in the present work of livers from rats fasted for 18 h, perfused with a saline medium containing no added substrates, seems reasonable.

Criteria of the condition of the liver during perfusion

An ideal criterion, as well as changing measurably at an early stage during the onset of any deterioration of condition, should (*a*) not be affected by the presence of different substrates, or alternatively should have a known value for a given set of conditions, and (*b*) should be applicable in all experimental perfusions, not only those designed to establish the suitability of the method. Of the measurements studied, initial recovery of potassium, ATP concentrations and lactate to pyruvate ratio showed significant differences between normal and 'unsatisfactory' perfusions (Table 4). Of these, ATP concentrations, or better, the total content and ratios of the adenine nucleotides, are probably among the most acceptable criteria when applied to basal perfusions, but in many experimental perfusions quite physiological changes in these values may be expected. The initial potassium recovery appeared to be affected relatively little by the presence in the perfusate of different substrates. Moreover, in blank perfusions the quantity of potassium recovered by the liver in the first 20–30 min of perfusion is related to the liver ATP concentrations after perfusion (Fig. 8). This is not surprising since, as mentioned earlier, the amount of potassium lost during the operation and transfer will depend on the degree of trauma sustained by the liver during these procedures, and the recovery of potassium – a measure of this loss – will therefore reflect the degree of success or otherwise of the subsequent perfusion. Further,

determination of perfusate potassium provides a continuous measure of the state of the liver during perfusion.

The lactate to pyruvate ratio changes markedly under different physiological conditions as well as during the onset of liver damage and its use as a criterion of viability is difficult to assess until more results are available, because its value has not yet been established for a particular set of experimental conditions. It is clearly not correct to suppose that measurements such as the lactate to pyruvate ratio should necessarily be the same in the perfused liver as in in vivo controls. In perfusions, substrate and hormonal conditions imposed on the liver are very different from those in vivo, even when rat blood is used as perfusion medium.

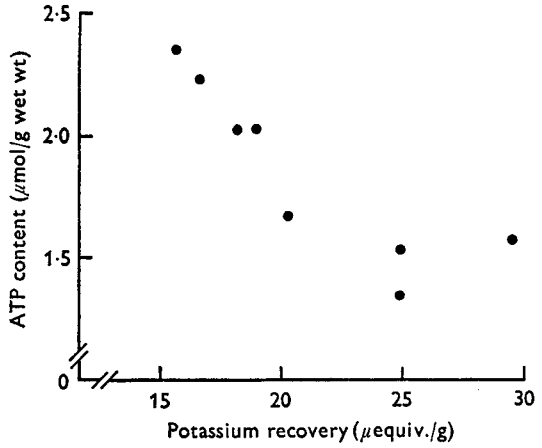

Fig. 8. Relation between the initial potassium recovery by the rat liver and the liver ATP content after blank perfusion. The livers were removed under halothane anaesthesia and perfused for 2 h.

In view of these considerations, in the work reported here the potassium measurements were taken as the most important routine criterion of the condition of the perfused liver, with water content after perfusion, bile flow-rate, perfusate flow-rate and general appearance as subsidiary criteria. The other measurements given in this paper are aimed at providing additional evidence that the perfusion method is capable of producing satisfactory perfusions.

The condition and performance of the perfused liver

The measurements of potassium and liver water content show that the liver recovers from the effects of the operation and transfer during the first 15–30 min of perfusion and that subsequently no deterioration is apparent throughout a 3 h perfusion. The rate of efflux of alanine aminotransferase from the liver cells was small, but these measurements are difficult to interpret precisely because it is not yet known at what rate it leaks from the tissues in vivo. There is evidence that plasma enzymes, including alanine aminotransferase, are turning over relatively rapidly in the intact animal and that the normal serum concentrations are maintained by leakage from the

tissues (Hess, 1963). The large normal range of plasma alanine aminotransferase activity in the rat (see p. 405) also suggests that in vivo efflux rates may vary considerably. Thus, although measurement of enzyme leakage from the liver is potentially a sensitive criterion of viability, it cannot usefully be applied until more information is available. However, the constant rate of efflux of alanine aminotransferase found in these experiments is evidence that there is no progressive deterioration of the liver during perfusion. The leakage rate is in agreement with that found by Spruyt from livers perfused with whole blood in the Fisher & Kerly (1964) perfusion system (J. E. L. Spruyt, personal communication), suggesting that the initial absence of protein in the saline perfusate does not result in a greater loss of intracellular enzymes. It should be noted, however, that denaturation of protein on the glass beads of the perfusate oxygenator in the system described here might possibly make measurements of perfusate enzymes suspect, though no evidence of denaturation on the beads has been encountered.

The ATP concentrations found compare favourably with those reported by others in livers perfused with media containing protein and red cells, though rather less than in vivo 'resting' values. The rates of bile secretion are similar to those found in vivo and with other perfusion systems; and oxygen consumption, rates of urea synthesis and glucose production correspond to reasonable extrapolations of results from livers perfused with a variety of media. The lactate to pyruvate ratios in livers after blank perfusions, while being difficult to interpret precisely, showed a more oxidized state in the cytoplasm than in vivo, which is in agreement with findings of other workers and is to be expected under the conditions of the perfusions (see p. 403). This itself is at least some indication that mitochondrial oxidation is not impaired and that the liver is not hypoxic, since Scholz *et al.* (1969) have shown that reduced pyridine nucleotide concentrations in the perfused rat liver are increased during anoxia.

The response of the liver to added amino acids seems satisfactory, and a comparison of the perfusate amino acid changes with changes in the blood of rats in vivo on passage through the liver (see Bloxam, 1972) suggests that the liver perfused with oxygenated Krebs–Ringer solution behaves from this point of view substantially as it does in vivo.

The output of glucose from 10 mM-lactate was within the range of rates found by other workers using established perfusion methods (Williamson *et al.* 1969; Struck *et al.* 1965; Exton & Park, 1967; Menahan *et al.* 1968; Ross, Hems, Freedland & Krebs, 1967; and see p. 407). Inspection of these references indicates that some of the differences in rates may be caused by differences in degree of starvation and in age and strain of the rats used. It is known, for instance, that the gluconeogenic capacity of the liver is increased in starvation, whether measured as net glucose synthesis from lactate (Exton, Jefferson, Butcher & Park, 1968; Ross *et al.* 1967) or as activity of the key gluconeogenic enzymes (Freedman & Kohn, 1964; Lardy, Foster, Shrago & Ray, 1964; Shrago, Lardy, Nordlie & Foster, 1963; Weber, 1959; Weber & Cantero, 1959) when related to the wet weight or protein content of the tissue; there is also an increase in the activities of hexosediphosphatase (EC 3.1.3.11) and phosphopyruvate carboxylase (EC 4.1.1.32) between 24 and 48 h of starvation (K. L. Manchester & J. Wimhurst,

personal communication). It is also known that the activities of enzymes of carbohydrate metabolism may vary widely in different strains and substrains of the rat (Eggleston & Krebs, 1969; Bartley, Dean, Taylor & Bailey, 1967). The possibility that unknown substances present in certain commercial preparations of the bovine serum albumin used in some perfusion media stimulate the rate of gluconeogenesis from lactate is mentioned by Williamson *et al.* (1969) and Exton & Park (1967).

Effects of anaesthetics

The most commonly used anaesthetics for liver perfusion work are pentobarbitone and diethyl ether. The results given here taken together with further work (Bloxam, 1967*a*) indicate that when pentobarbitone is used as the anaesthetic for the operation procedure subsequent bile secretion and urea and glucose production are inhibited to some extent. Pentobarbitone is also theoretically suspect because it is taken up and metabolized by the liver (Mark, 1963; Richards & Taylor, 1956), and because it is generally given intraperitoneally so that the liver probably receives a relatively undiluted dose. Gaseous anaesthetics have the following advantages over the barbiturates: they do not reach the liver in especially large amounts when taken in via the lungs, their dose can be varied at any time according to the appearance of the animal, and they are rapidly removed via the perfusate oxygenator during perfusion. Ether, however, has a number of effects on carbohydrate, amino acid and fat metabolism and also some hormonal effects (Bunker, 1962; Brunner, 1969; Marquez-Julio & French, 1968; Ngai & Papper, 1962; Schotz & Olivecrona, 1966), but halothane has the advantages of the gaseous anaesthetics without some of the disadvantages of ether (Bloxam, 1967*a*; Ngai & Papper, 1962; Vandam, 1966), and in addition is strongly vasodilatory so that the flow-rate through the liver is maximal. Halothane therefore seems the best anaesthetic to use for this type of work.

Control of perfusate amino acid concentration

The results from the experiments shown in Figs. 5 and 6 suggest that the perfused liver of the rat starved overnight can regulate the perfusate concentrations of the amino acids, except the branched-chain group, in either direction, by net movement into or out of the cells according to the perfusate concentrations. Published evidence indicates that this property is not shown by the transport systems of other tissues. See, for instance, reviews of Quastel (1965–6), Wilson (1962) and Christensen (1962, 1964).

The perfused rat liver has already been found to release amino acids into a perfusion medium initially free from amino acids, by Schimassek & Gerok (1965) and Mondon & Mortimore (1967) using livers of fed rats, and recently by Mallette, Exton & Park (1969) with livers of rats starved overnight. The perfusate concentrations found by this latter group of workers are in general agreement with those reported here, except that they observed a continuous increase in the levels of valine, leucine and isoleucine only when glucagon was added to the perfusion medium. The progress of uptake of amino acids by the liver in the work now described and the different behaviour of the branched-chain acids are substantially in agreement with the results of Fisher &

Kerly (1964) and of Spruyt (1964) from rat livers perfused with rat blood. A comparison of the equilibrium concentrations found in blank perfusions and the plasma concentrations in blood from rats starved for 18–20 h, of the same weight and type as the liver donors for the perfusions, is shown in Table 7. The perfusate concentrations in all instances (except for valine, leucine and isoleucine) were lower than the blood levels. If, as these results suggest, the liver in vivo tends to regulate concentrations of blood amino acid levels below those actually found, since plasma concentrations in vivo remain relatively constant, there must be net flow of most of the amino acids from extrahepatic tissues to the liver, whilst valine, leucine and isoleucine flow

Table 7. *Comparison between amino acid equilibrium concentrations of perfusates from blank perfusion of livers from rats starved for* 18–20 *h and plasma concentrations of rats starved for* 18–20 *h*

(Mean values with their standard errors; the perfusate values are from five perfusions and the plasma values are from eight animals)

Amino acid	Perfusate concentration (μmol/100 ml)	Plasma concentration (μmol/100 ml)
Taurine	5·5 ± 1·0	14·2 ± 1·0
Aspartate	0·2 ± 0·0	1·0 ± 0·1
Threonine	6·2 ± 0·9	30·3 ± 2·7
Serine	2·1 ± 0·2	45·8 ± 4·1
Glutamate	3·0 ± 0·4	4·2 ± 0·3
Proline	1·4 ± 0·1	14·2 ± 0·7
Citrulline	1·2 ± 0·1	7·0 ± 0·3
Glycine	12·8 ± 1·0	43·7 ± 2·5
Alanine	1·0 ± 0·2	33·4 ± 1·6
Cystine	0·3 ± 0·1	0·9 ± 0·2
Methionine	2·4 ± 0·1	6·1 ± 0·2
Tyrosine	6·9 ± 1·2	8·5 ± 0·8
Phenylalanine	4·2 ± 0·2	7·4 ± 0·4
Ornithine	3·6 ± 0·5	8·1 ± 0·6
Lysine	9·4 ± 1·0	48·6 ± 4·2
Histidine	5·1 ± 0·2	7·1 ± 0·3
Tryptophan	0·8 ± 0·1	8·7 ± 0·9
Arginine	0·7 ± 0·1	8·6 ± 0·7
Valine	—	23·1 ± 1·1
Leucine	—	21·7 ± 1·1
Isoleucine	—	14·4 ± 0·7

in the opposite direction, from the liver to peripheral tissues where they are oxidized more rapidly, by extrahepatic tissues as a whole (Miller, 1962), by adipose tissue (Feller, 1965; Feller & Feist, 1959–60), by kidney (Dawson, Hird & Morton, 1967), by diaphragm muscle (Manchester, 1965) and by heart muscle (Clarke, 1957). This suggestion is supported by the finding that the concentrations of most amino acids in the blood decrease on passage through the liver in the intact dog (Elwyn, 1966), in man (Carlsten & Werkö, 1967) and through the isolated rat liver (Fisher & Kerly, 1964) and also by the fact that when the liver is removed from the dog and the rat there is an increase in blood amino acid concentrations, again except for valine, leucine and isoleucine which do not increase, or actually decrease (Flock, Mann &

Bollman, 1951; Freeman & Svec, 1951; McMenamy, Vang & Drapanas, 1965; Miller, 1962). The results also suggest that there could be an efflux of amino acids from the liver, tending to maintain blood concentrations, under certain conditions, such as during prolonged starvation or during a diet imbalanced with respect to amino acids.

The quantity of the branched-chain acids given out by the liver during the perfusions is considerably greater than that initially present in the intracellular free amino acid pool (Bloxam, 1972). Their continuous output must therefore be derived from breakdown of liver protein since, being essential, they are not synthesized to an appreciable extent in the liver. This is consistent with the fact that the liver of the rat starved overnight is probably losing protein relatively rapidly (Munro, 1964; Addis, Poo & Lew, 1936*a*, *b*, *c*). It therefore seems likely that the output of these amino acids would revert to an uptake during the period of net protein synthesis which probably occurs in the liver after feeding (Munro, 1964; Addis *et al.* 1936*c*; Potter, Baril, Watanabe & Whittle, 1968), and some evidence for this is found in the next paper in the series (Bloxam, 1972).

The depression of the perfusate concentrations of some amino acids, most marked for tyrosine, glycine and lysine (Fig. 5), after an initially high perfusate concentration of amino acid mixture has an interesting parallel in work done by Frame (1958), who gave a high-protein meal to human subjects and found that after the expected rise in plasma amino acid concentrations some fell to concentrations below the original fasting values, before these were finally re-established. Moreover, Hunter, Laastuen & Todd (1967) have found that feeding glycine to rats caused a depression of some amino acids, notably tyrosine. These could be manifestations of the same effect in vivo, additional to the known lowering of plasma amino acid concentrations (see Manchester, 1970) by insulin, whose secretion is stimulated by administration of amino acids (Floyd, Fajans, Conn, Knopf & Rull, 1966).

Conclusion

The results given here show that the liver can be maintained with oxygenated Krebs–Ringer bicarbonate buffer in apparently as good a condition as livers perfused with media containing protein and red cells, with or without substrates. Enough oxygen is available in the preparation described here for the liver to deal with large amounts of added substrate.

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