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# Gluconeogenesis in the Perfused Rat Liver

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1. A modification of the methods of Miller and of Schimassek for the perfusion of the isolated rat liver, suitable for the study of gluconeogenesis, is described. 2. The main modifications concern the operative technique (reducing the period of anoxia during the operation to 3min.) and the use of aged (non-glycolysing) red cells in the semi-synthetic perfusion medium. 3. The performance of the perfused liver was tested by measuring the rate of gluconeogenesis, of urea synthesis and the stability of adenine nucleotides. Higher rates of gluconeogenesis (1 $\mu$mole/min./g.) from excess of lactate and of urea synthesis from excess of ammonia (4 $\mu$moles/min./g. in the presence of ornithine) were observed than are likely to occur *in vivo* where rates are limited by the rate of supply of precursor. The concentrations of the three adenine nucleotides in the liver tissue were maintained within 15% over a perfusion period of 135min. 4. $Ca^{2+}$, $Na^{+}$, $K^{+}$, $Mg^{2+}$ and phosphate were found to be required at physiological concentrations for optimum gluconeogenesis but bicarbonate and carbon dioxide could be largely replaced by phosphate buffer without affecting the rate of gluconeogenesis. 5. Maximal gluconeogenesis did not decrease maximal urea synthesis in the presence of ornithine and ammonia and vice versa. This indicates that the energy requirements were not limiting the rates of gluconeogenesis or of urea synthesis. 6. Addition of lactate, and especially ammonium salts, increased the uptake of oxygen more than expected on the basis of the ATP requirements of the gluconeogenesis and urea synthesis.

Attempts in this Laboratory to study gluconeogenesis in rat liver slices met with major difficulties: the rates of gluconeogenesis obtained with substrates such as succinate, malate, fumarate, glutamate and aspartate were unexpectedly low or even nil. Lactate and pyruvate formed glucose but less rapidly than expected. These observations indicate that liver slices have a limited scope in the study of gluconeogenesis and it was therefore decided to use the isolated perfused rat liver.

Earlier workers (for reviews see Baglioni, 1910; Kapfhammer, 1927; Kestens, 1964) perfused livers of larger animals, mainly dogs, because the surgical and analytical chemical techniques available at that time were on the 'macro' scale. The first to use the perfused rat liver were Corey & Britton (1941), Trowell (1942), Brauer, Passotti & Pizzolato (1951) and Miller, Bly, Watson & Bale (1951). The method of Miller *et al.* (1951) has proved to be the most acceptable one to subsequent workers (see e.g. Fisher & Kerly, 1964) and this technique, with modifications introduced by Mortimore (1961) and Schimassek (1963*a*), was the starting point of the present investigation. Miller *et al.* (1951) perfused the isolated liver from the portal vein with heparinized rat blood under a pressure of about 20cm. water, maintained by a reservoir of adjustable height. The blood leaves through the vena cava and drops into a collecting vessel. From here it is pumped to the top of a multiple-bulb oxygenator and then returned into the reservoir. Mortimore perfused the liver *in situ*, but isolated with respect to the circulation from the donor animal. Schimassek (1963*a*) replaced the blood by a saline medium supplemented by bovine albumin and bovine erythrocytes.

Preliminary experiments with the procedure of Schimassek showed that the perfused liver forms glucose from lactate at somewhat higher rates than do slices but the same substances which failed to be glucogenic in slices also gave negative results in the perfused organ. This led to a systematic investigation of the perfusion technique with the object of improving it. To assess the efficiency of any modifications it was necessary to adopt objective criteria. Previous authors relied on the macroscopic and histological appearance of the liver, the oxygen consumption, the oxygen content of the outflowing blood and the rate of bile production. A major additional criterion used in the

present work is the synthetic capacity of the perfused organ as measured by the rate of gluconeogenesis from lactate and by the rate of urea formation from ammonium salts. Synthetic functions suffer very early when a tissue deteriorates and, in liver, the most exacting syntheses in terms of ATP requirements are gluconeogenesis and urea formation. Six moles of ATP are required for the synthesis of 1 mole of glucose from lactate and 4 moles of ATP for the synthesis of 1 mole of urea. Since the rates of glucose and urea synthesis can each exceed 1 $\mu$mole/g. fresh wt./min. the rates of the ATP requirement in the presence of lactate and ammonium salts are high enough to claim the greater part of the total energy supply of the liver.

## EXPERIMENTAL

*Analytical methods.* Glucose was determined by the glucose oxidase method as described by Krebs, Bennett, de Gasquet, Gascoyne & Yoshida (1963) and Krebs, Dierks & Gascoyne (1964). L(−)-Lactate was determined with lactate dehydrogenase according to Hohorst (1963). Urea was measured by the method of Conway (1962), ammonia by nesslerization. For the analysis of liver samples a lobe was rapidly frozen with the clamps of Wollenberger, Ristau & Schoffa (1960). About 1 g. of frozen material was ground in a cooled percussion mortar (Newsholme, 1962) and the powder was further ground with 3 ml. of frozen 6% (w/v) $HClO_4$ (Lowry, Passonneau, Hasselberger & Schulz, 1964). After thawing, centrifugation and removal of the $HClO_4$ with KOH, the concentrations of ATP, ADP and AMP were measured by the methods of Adam (1963) and Lamprecht & Trautschold (1963).

Haemoglobin was determined as cyanmethaemoglobin (see Standards Committee Report, 1965; Wintrobe, 1961). The total oxygen content of the perfusion fluid (oxyhaemoglobin and dissolved $O_2$) and the total $CO_2$ content (free $CO_2$ and $HCO_3^-$) were determined with the micro-gasometer of Natelson (1951). When the perfusion medium contained no haemoglobin, the oxygen content was measured with the oxygen electrode of Bishop (1960), obtained from Electronics Instruments Ltd., Richmond, Surrey. It was directly coupled to an amplifying recorder (Kipp micrograph BD2 of Kipp, Delft, Holland). The electrode was fitted into a small glass chamber (2 ml. capacity) provided with a magnetic stirrer, Y tubes and clips which made it possible to divert samples of either the 'arterial' or 'venous' perfusion medium through the measuring chamber. From this chamber the medium was returned to the collecting vessel (see Fig. 1). The arrangement supplied a continuous record of the venous $O_2$ tension.

Before and after each experiment the electrode was calibrated at 35° against (1) water saturated with 100% $O_2$, (2) with air (21% $O_2$), and (3) aq. dithionite (zero $O_2$).

*Perfusion medium.* A medium, among many tried, which gave optimum rates of gluconeogenesis contained: (1) physiological saline (Krebs & Henseleit, 1932), (2) bovine serum albumin powder fraction V (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) and (3) washed human red cells. Since the study of gluconeogenesis was the major aim of the current work it was undesirable to have glycolysing red cells in the perfusion system. For this reason aged human blood, stored 4–5 weeks at 4° and no longer suitable for blood transfusion, was obtained from the United Oxford Hospital Blood Transfusion Centre. This still possessed the full $O_2$-carrying capacity but did not glycolyse, as regular checks showed. To remove the plasma, added citrate, residual glucose and the accumulated lactate, 100 ml. of whole blood was centrifuged at 2000 *g* for 10 min. and the plasma and buffy layer were removed by suction. The cells were washed three times with about 3 vol. of the physiological saline medium gassed with $CO_2+O_2$ (5:95). The washing fluid was removed by centrifugation for 5 min. at 2000 *g*. The washed cells were made up to 100 ml. with the saline to give a haemoglobin concentration between 10 and 15 g./100 ml. This stock suspension was used on the day of preparation and kept at 4°. On storage beyond 24 hr. in the absence of glucose the cells are liable to lyse.

For one perfusion 150 ml. of medium was needed. This was prepared from the above three stock reagents by dissolving, with stirring, 3·9 g. of bovine albumin in about 100 ml. of saline medium. Since the albumin was acid, the pH had to be adjusted to 7·4 by the addition of about 0·5 ml. of N-NaOH (controlled by a glass electrode). Then enough of the red-cell suspension was added to give a final haemoglobin concentration of 2·5 g./100 ml. Further alkali in the form of M-$NaHCO_3$, usually 2 ml., was added to bring the pH to 7·3–7·4 and the bicarbonate concentration to 25 mM. The latter was checked in the Natelson apparatus after equilibrating the medium with $CO_2+O_2$ (5:95). Finally sufficient saline was added to bring the total volume to 150 ml.

A haemoglobin concentration of 2·5 g./100 ml. was chosen because it was adequate at the fast flow rates used. It provided a safety margin for the oxygenation of the tissue and yet allowed a reliable measurement of the arteriovenous $O_2$ difference. Usually about half the $O_2$ entering the tissue was used. At physiological haemoglobin concentration it was not possible to measure the consumption of $O_2$ accurately. An additional advantage of the low red cell content was the relatively low viscosity of the medium which permitted a more rapid flow.

When substrates were to be added a fraction of the saline, usually 7·5 ml., was replaced by a 0·2 M neutral substrate solution. The final concentration of the albumin was 2·6% (w/v), of haemoglobin 2·5% (w/v) and of substrates (usually) 10 mM.

*Perfusion apparatus.* The apparatus, based on the designs of Miller *et al.* (1951) and of Schimassek (1963*a*), is housed in a cabinet provided with sash Perspex windows at the front and rear and is heated by a thermostatically controlled fan heater. The temperature of the cabinet and of the circulating medium was 35°. At 40° the performance of the liver tended to deteriorate, as indicated by a patchy colouring, towards the end of the usual perfusion period of about 2 hr. There is enough room in the cabinet (45 cm. × 85 cm. × 100 cm.) for two simultaneous perfusions, with access from opposite sides. Magnetic stirrers are placed below the floor of the cabinet to mix the perfusion medium in the collecting vessels and provision is made for gas to be supplied from cylinders outside the cabinet.

The design of the glassware and platform for supporting the animal and the arrangement of the apparatus are shown in Fig. 1. The multi-bulb glass oxygenator is attached to a stainless-steel plate which is mounted on a

ball-and-socket joint and clamped to a vertical rod. The joint makes it possible to adjust the position of the oxygenator accurately to a vertical one which ensures complete spread of the perfusion fluid over the oxygenator's surface. At the bottom of the oxygenator is a reservoir containing oxygenated medium. The height of the reservoir is adjusted to give a sufficient hydrostatic pressure for optimum flow rates without swelling of the liver. This pressure varies between 20 and 30 cm. from liver to liver.

The perfusion medium is pumped from the collecting vessel by a type MHRE roller pump supplied by Watson (Marlow) Ltd., Marlow, Bucks. It passes through a plastic mesh filter, taken from a disposable blood transfusion set (Capon, Heaton and Co. Ltd., Birmingham), which is changed every few days. From the filter the fluid moves to the top of the oxygenator. The gas is usually $CO_2 + O_2$ (5:95). It is saturated with water by bubbling through a wash bottle fitted with a sintered-glass distributor. It enters the oxygenator at the bottom and leaves through an outlet at the top. The reservoir where the oxygenated medium is collected has an overflow by which the excess of medium is returned to the collecting vessel. AB 14 standard ground-glass joint at the bottom of the oxygenator fits a thermometer or a combination pH electrode.

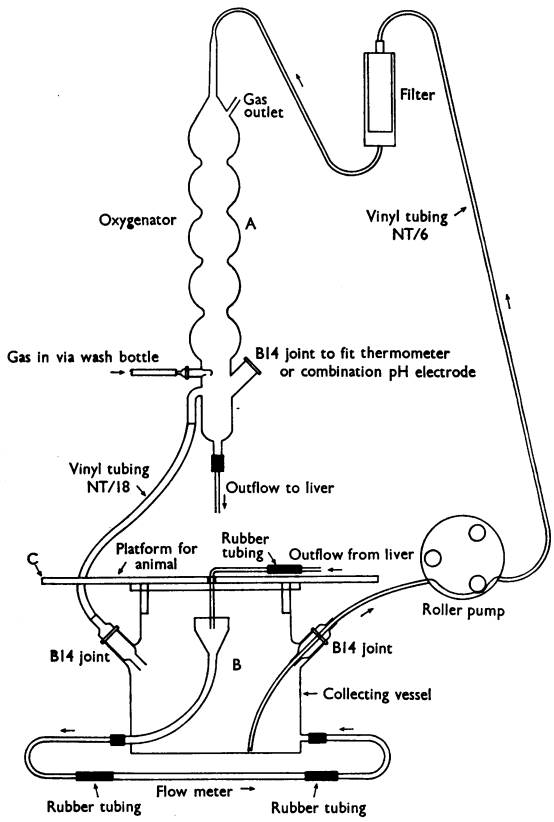


Fig. 1. Apparatus for the perfusion of rat liver. For details see the text. The diameter of the bulbs of the oxygenator (A) is 40 mm. The diameter and height of the collecting vessel (B) are 80 mm. and 100 mm. respectively. The supporting platform (C) for the animal is 250 mm. by 150 mm.

The collecting vessel is provided with a flanged top on which the Perspex platform rests. A Perspex ring, fixed underneath the platform and fitting closely inside the collecting vessel, gives stability to the platform. A centrally placed hole in the platform takes a tube through which the outflowing perfusion medium from the liver returns to the collecting vessel via a funnel. The outlet of this is fused into the wall of the collecting vessel. The medium then passes through a flow meter. The flow rate is measured by injecting an air bubble into the rubber tubing at the beginning of the flow meter and timing its travel along the 5 ml. calibrated glass tube.

Transparent vinyl tubing no. NT/6 (Portland Plastics Ltd., Hythe, Kent) is used throughout except for the piece connecting the overflow of the reservoir with the collecting vessel, where a wider gauge NT/18 tubing is used to prevent the formation of bubbles, and a short piece of rubber tubing is used at the beginning of the flow meter.

The thin transparent vinyl tubing is permeable to $O_2$ and other gases. When gas exchanges are to be measured the relevant tubing is replaced by glass tubing of the same diameter consisting of short lengths, joined together, almost glass to glass, by vinyl tubing to give flexibility.

In addition to the two B14 openings on the reservoir flask shown in the Figure there is another B14 joint (not shown in the Figure) at the front of the reservoir through which samples of medium can be removed for analysis.

The total volume of the perfusion medium is 150 ml., a volume which is convenient from the point of view of the magnitude of many metabolic changes. Thus 1500 $\mu$moles of lactate (10 mM) can be quantitatively converted into glucose in about 90 min. This is accompanied by an equivalent formation of $HCO_3^-$ which would cause a major pH change in a small volume. When the synthesis of urea from $NH_4^+$ is studied there is an equivalent acid production which has to be neutralized. If in other studies where metabolic changes are smaller less perfusion medium is desirable, the shape of the collection vessel can be modified.

*Operative technique.* The rat is anaesthetized for the operation by intraperitoneal injection of Nembutal (0·1 ml. of 6% solution per 100 g. body wt.). Then heparin (0·1 ml. = 100 units) is injected into the saphenous vein. The abdomen is opened through a mid-line incision and mid-transverse incisions to left and right of the mid-line are made, avoiding the larger vessels. Bleeding is minimized by clamping the major vessels of the abdominal wall with four artery forceps. The intestines are then placed to the animal's left, between layers of tissue wetted with saline, so that the liver, portal vein, right kidney, inferior vena cava and the bile duct become exposed. The thin strands of connective tissue between the right lobe of the liver and the vena cava are cut and a loose ligature of silk (size 3/0) is placed around the cava above the right renal vein.

Next the bile duct is cannulated by a 30 cm. length of Portex tubing size PP10 cut at an angle to provide a sharp point. The cannula is inserted and pushed to the point where the duct arises from its branches and secured with a silk ligature.

Three loose ligatures are passed around the portal vein at intervals of 3–4 mm. below the point where the vein

divides to enter the separate lobes of the liver and a fourth ligature is placed around the vein at a point distal to the liver. The portal vein is then cannulated with a no. 16 Frankis–Evans needle (trocar and cannula, Luer fitting). With practice and care the blood loss is negligible. The three loose ligatures are tied, securing the cannula in place, and the fourth ligature is tied, shutting off the blood supply from the viscera to the portal vein. At this stage the inside needle is removed, leaving the cannula in position.

The thorax is opened by a transverse incision just above and along the line of the diaphragm and by two longitudinal incisions towards the head from the two ends of the transverse incision. The chest wall is flapped back towards the head and a large (15 cm.) pair of artery forceps is placed along the base of the flap and locked in position. The flap is then cut off. The vagus and phrenic nerves and oesophagus are cut about 1 cm. above the diaphragm to paralyse the diaphragm and to eliminate possible vasoconstrictor effects of the vagus, and a loose ligature is placed around the inferior vena cava close to the heart. The cannula that is placed in the inferior vena cava consists of a 5 cm. length of Portex tubing PP270 which has been heated in a gas flame, drawn out to an outside diameter of 2 mm. and cut off at an angle to form a sharp tip. This is sharp enough to penetrate the right atrium: it is pushed as far down the vein towards the diaphragm as it will go and then tied in position. At this stage the loose ligature around the abdominal vena cava is tied. The cannula lying in the portal vein has usually filled with blood but if this is not the case perfusion medium is injected with a syringe through the inner needle of the cannula. Care is taken to avoid admission of air into the portal vein; the medium is injected continuously while withdrawing the needle.

The preparation is then connected to the perfusion apparatus and the circulation started at a flow rate of 15–25 ml./min. The first 5 ml. of venous blood is discarded.

The whole operation takes about 10 min. There is an interval of about 2·5 min. from the introduction of the cannula into the portal vein, during which the circulation is impaired, and a period of about 1 min. between the cessation of breathing caused by the opening of the thorax and the start of the perfusion. An indication of the success of the operation is a uniform red colour of the perfused liver, indistinguishable from the colour *in situ*. An extended interruption of the liver circulation may cause an initial patchy perfusion, indicated by uneven colouring from which the liver may not completely recover.

*Sampling of perfusion fluid.* For the determination of oxygen in the perfusion fluid, inflowing oxygenated samples are taken with a glass syringe from the bottom of the reservoir, and outflowing samples from the rubber link in the tubing system leaving the vena cava. As storage in the syringe may lead to changes in the gas content, the samples are introduced into the micro-gasometer without delay. The $O_2$ content of the oxygenated sample does not vary significantly during a single experiment ($P > 0·5$ for 18 pairs of readings). It is therefore not essential to collect the oxygenated sample at the same time as the venous. Oxygenated samples were usually taken at 15 and 75 min. and venous samples at 30 and 60 min. in experiments where substrate was added at 38 min. The flow rate was measured at the time of venous sampling.

For other chemical analyses samples of the medium (0·5 ml.) are taken from the collecting vessel and pipetted directly into 4 ml. of 2% $HClO_4$. The resulting small changes in the volume of the perfusing fluid are taken into account in the calculations of the metabolite content of the medium.

Usually an initial sample is taken before the perfusion starts to measure the small quantities of glucose, lactate and other metabolites present in the fluid. Other samples are taken after 10 and 40 min. Within the first 40 min. any glucose formed from glycogen present in the liver initially and from lactate left in the washed red cells has appeared in the medium. The slow continuing rate of glucose appearance (0·15 μmole/g./min.) is the result of gluconeogenesis from endogenous precursors. The substrate is added at 38 min. The interval between 38 and 40 min. is adequate for mixing the substrate with the whole perfusion fluid as about 50 ml. of the fluid is in circuit at any one time and the pump output via the liver and overflow is about 170 ml./min. Subsequent samples are usually taken every 15 min. for 90 min.

## RESULTS

### *Performance of the perfused liver*

*Time-course of gluconeogenesis from lactate.* Representative records of a control perfusion to which no substrate has been added and a perfusion with 10 mM-L-lactate obtained with livers of rats starved for 48 hr. (Table 1) show that the starved liver shed appreciable amounts of glucose in the

Table 1. *Time-course of gluconeogenesis from lactate in the perfused rat liver*

Livers of rats starved for 48 hr. were perfused as described in the text. The wet wt. of both livers was 5·7 g., the dry wt. 1·63 g.

| Time (min.) ... | 0 | 10 | 40 | 55 | 70 | 85 | 100 | 115 | 130 |
|---|---|---|---|---|---|---|---|---|---|
| Expt. 1 (no substrate added) | | | | | | | | | |
| Glucose found (μmoles in total perfusion medium) | 50 | 108 | 152 | 174 | 179 | 214 | 216 | 206 | 214 |
| Lactate found (μmoles in total perfusion medium) | 155 | 170 | 173 | 168 | 154 | 146 | 154 | 163 | 145 |
| Expt. 2 (1500 μmoles of L-lactate added to 150 ml. of medium at 38 min.) | | | | | | | | | |
| Glucose found (μmoles in total perfusion medium) | 33 | 42 | 57 | 120 | 225 | 330 | 420 | 550 | 640 |
| Change in glucose (μmoles/15 min. interval) | | | | +63 | +105 | +105 | +90 | +130 | +90 |
| Lactate found (μmoles in total perfusion medium) | 132 | 138 | 1621 | 1464 | 1246 | 1128 | 880 | 695 | 500 |
| Change in lactate (μmoles/15 min. interval) | | | | −157 | −218 | −118 | −248 | −185 | −195 |

first 10min. and rapidly decreasing amounts subsequently. Between 40 and 85min. the average rate of glucose formation was about 0·24μmole/min./g. and afterwards the glucose formation ceased. The average rate between 40 and 130min. (i.e. the period for which the effects of added substrate was measured) was 0·12μmole/min./g. The concentration of lactate, initially about 1mM, rose slightly in the first 40min. and then fell slowly. At 130min. it was again about 1·0mM. In some experiments it fell to lower values. When rats were starved for less than 48hr., larger quantities of glucose were released, both early and after 40min.

Addition of L-lactate after 38min., when the main endogenous glucose formation had stopped, caused a greatly increased output of glucose. After a short lag period the average rate of glucose formation was in this experiment 1·15μmoles/min./g. Lactate was removed at a rate a little below twice the rate of glucose formation (2·2μmoles/min./g.). These results are in agreement with the conclusion that conversion into glucose was the main fate of lactate and that in the presence of lactate small amounts of glucose were still formed from endogenous precursors.

The measurements of the changes in 15min. periods showed some variations owing to the experimental errors arising from the determination of relatively small differences. The rates of glucose formation and lactate removal were therefore always calculated from the gradient of the plot of the time-course after the lag period. As a rule this was linear.

No glycogen was deposited in the liver under the conditions of this experiment. In several cases a small liver lobe was removed at 40 and 130min. The glycogen content at 40min. was 1·02±0·23 (4) μmoles/g. and at 130min. 0·85±0·30 (4) μmole/g.

*Time-course of ammonia removal and urea synthesis.* Similar experiments were carried out to measure urea formation from added ammonium salts (Table 2). In the control experiment where no ammonia was added the concentration of ammonia remained near the initial value of about 0·5mM. The rate of urea formation during the first 40min. when some of the liver urea was washed out was 0·12μmole/min./g. and thereafter about 0·1μmole/min./g. Addition of 10mM-ammonium chloride led to a rapid removal of ammonia from the medium, at the rate of 1·4μmoles/min./g., and urea appeared in the medium at a steady rate (0·8μmole/min./g.). Thus all the ammonia removed could be accounted for by the formation of urea.

Addition of both ammonium chloride (10mM) and ornithine (2·7mM) caused a great increase in the rates of ammonia removal and urea formation, as expected. During the 40–90min. period the rate of ammonia removal was 4·5μmoles/min./g. In the following 45min. the rate fell but when more ammonium chloride was added after 143min. the earlier high rates of ammonia removal and urea synthesis were almost completely restored (Table

Table 2. *Time-course of urea formation and ammonia removal in the perfused rat liver*

Livers of rats starved for 48hr. were perfused as described in the text. Weights of the livers were 5·8, 5·1 and 6·3g. in Expts. 1, 2 and 3 respectively.

| | Time (min.) ... | 0 | 10 | 40 | 55 | 70 | 85 | 100 | 115 | 130 |
|---|---|---|---|---|---|---|---|---|---|---|
| Expt. 1 (no substrate added) | $NH_3$ found (μmoles in total medium) | 87 | 97 | 99 | 90 | 87 | 80 | 81 | 87 | 97 |
| | Urea found (μmoles in total medium) | 33 | 46 | 61 | 68 | 78 | 84 | 89 | 98 | 110 |
| Expt. 2 (1500μmoles of $NH_4Cl$ added at 38min.) | $NH_3$ found (μmoles in total medium) | 80 | 90 | 1570 | 1450 | 1350 | 1270 | 1140 | 1030 | 930 |
| | $NH_3$ removed/15min. interval (μmoles) | | | | −120 | −100 | −80 | −130 | −110 | −100 |
| | Urea found (μmoles in total medium) | 20 | 28 | 35 | 102 | 155 | 207 | 280 | 340 | 393 |
| | Urea formed/15min. interval (μmoles) | | | | +67 | +53 | +52 | +73 | +60 | +53 |
| Expt. 3 (1500μmoles of $NH_4Cl$ and 400μmoles of ornithine hydrochloride added at 38min.) | $NH_3$ found (μmoles in total medium) | 135 | 150 | 1650 | 1260 | 800 | 560 | 460 | 383 | 300 |
| | $NH_3$ removed/15min. interval (μmoles) | | | | −390 | −460 | −240 | −100 | −77 | −83 |
| | Urea found (μmoles in total medium) | 40 | 46 | 60 | 264 | 502 | 630 | 695 | 746 | 801 |
| | Urea formed/15min. interval (μmoles) | | | | +204 | +238 | +128 | +65 | +51 | +55 |

Table 3. *Time-course of urea synthesis in the presence of ornithine after a second addition of ammonium chloride*

A second dose of 1500 $\mu$moles of $NH_4Cl$ was added at 143 min. to the perfusion recorded in Table 2, Expt. 3.

| Time (min.) ... | 145 | 160 | 175 | 190 |
|---|---|---|---|---|
| $NH_3$ found in total medium ($\mu$moles) | 1750 | 1355 | 945 | 600 |
| Change in $NH_3$ ($\mu$moles/15 min.) | | −395 | −410 | −345 |
| Urea found in total medium ($\mu$moles) | 820 | 1020 | 1235 | 1420 |
| Change in urea ($\mu$moles/15 min.) | | −200 | −215 | −185 |

Table 4. *Performance of the perfused rat liver as tested by gluconeogenesis from lactate and urea synthesis from ammonia*

Livers of rats starved for 48 hr. were perfused as described in the text. The quantities of lactate and ammonia added in 150 ml. of perfusion fluid were 1500 $\mu$moles and that of ornithine was 400 $\mu$moles. The results are $\mu$moles/g. wet wt./min. (mean ± S.E.M. and, in parentheses, the number of observations). The extra $O_2$ used was calculated in each experiment from the measurement of the $O_2$ uptake before and after addition of substrates. The extra $O_2$ uptake expected for meeting the extra ATP needs was calculated on the assumption that the synthesis of 1 glucose from lactate requires 1 $O_2$ (6 ATP) and the synthesis of 1 urea from $NH_3$ requires 4 ATP ($\frac{2}{3}$ $O_2$).

| Substrates added | Glucose formed | Lactate used | Urea formed | $O_2$ used | Extra $O_2$ used on addition of substrates | Extra $O_2$ uptake calc. for ATP needs |
|---|---|---|---|---|---|---|
| None | 0·14 ± 0·026 (5) | — | 0·09 ± 0·01 (4) | 2·20 ± 0·22 (12) | — | — |
| L-Lactate | 1·06 ± 0·09 (12) | 1·95 ± 0·23 (12) | — | 3·50 ± 0·20 (5) | 1·3 ± 0·22 | 0·92 |
| $NH_4Cl$ | — | — | 0·80 ± 0·14 (4) | 3·64 ± 0·18 (4) | 1·66 ± 0·34 | 0·48 |
| L-Lactate; $NH_4Cl$ | 1·05 ± 0·06 (4) | 2·24 ± 0·11 (4) | 0·45 ± 0·04 (4) | 6·02 ± 0·28 (4) | 3·44 ± 0·18 | 1·25 |
| $NH_4Cl$; L-ornithine | 0·185 ± 0·05 (5) | — | 1·87 ± 0·27 (5) | 4·60 ± 0·40 (4) | 2·60 ± 0·14 | 1·19 |
| $NH_4Cl$; L-ornithine; L-lactate | 1·05 ± 0·05 (5) | 2·19 ± 0·17 (5) | 1·95 ± 0·26 (5) | 8·00 ± 0·80 (5) | 5·60 ± 0·93 | 2·15 |

3). The average rate of ammonia disappearance for the period between 145 and 190 min. was 4·05 $\mu$moles/min./g. This indicated that the fall in the concentration of ammonium chloride was solely responsible for the fall in the rate observed after 90 min.

The rate of urea formation was slightly higher than half the rate of ammonia removal. Thus some urea was formed from endogenous sources. However, in the presence of ornithine when the rate of urea formation from added ammonia was greatly increased the discrepancy was slight and in further experiments it was thought to be sufficient to measure only the rate of ammonia removal.

*Biosynthetic capacity of perfused liver*

The biosynthetic capacity of the liver was tested by measuring the maximum rates of gluconeogenesis from lactate and urea synthesis under optimum substrate conditions (Table 4). Lactate, or ammonium chloride, or ornithine plus ammonium chloride, or these three substrates together, were added. The maximal rates were obtained from the linear section of the time-course. The uptake of oxygen was measured at 30 min. (i.e. before addition of substrates) and at 60 min. (i.e. 20 min. after addition of substrates) when urea synthesis and gluconeogenesis occurred at the maximal rates.

Although both processes require ATP, their rates were additive when lactate, ammonium chloride and ornithine were present. Thus gluconeogenesis did not inhibit maximal rates of urea synthesis and vice versa. In the absence of ornithine, however, lactate decreased the rate of urea synthesis to almost half. This inhibition must have been due to factors other than energy requirements. The observed rates of gluconeogenesis from lactate were similar to those of Exton & Park (1965), who found 1·0 ± 0·3 (19) $\mu$mole/min./g. under comparable conditions, and somewhat higher than those of Struck, Ashmore & Wieland (1965), who found 0·77.

The consumption of oxygen was 2·2 $\mu$moles/min./g. when no substrate was added, a value rather higher than those found for the perfused starved liver by Forsander, Räihä, Salaspuro & Mäenpää (1965) and Schnitger, Scholz, Bücher & Lübbers (1965). The addition of the substrates caused an increased oxygen consumption in every case. This

increase was somewhat greater than expected on the basis of the extra energy requirements of gluconeogenesis when lactate alone was added. It was substantially in excess of the expected value when ammonium chloride alone or together with lactate or with ornithine or with lactate and ornithine was present (compare last two vertical columns of Table 4).

The value for the uptake of oxygen of 2·2 $\mu$moles/min./g. without added substrate is comparable with that found in slices ($Q_{O_2}$ 10) but the high rates found on addition of lactate, ammonium chloride and ornithine (8·0 $\mu$moles) are about twice as high as the highest rates observed with slices under comparable conditions (see Krebs, 1934, 1950).

*Effect of modifications of the perfusion medium.* Many experiments were carried out in which modifications of the medium were tested, with the adequacy of flow through the liver and the rate of glucose formation from lactate as criteria. Omission of the albumin caused gross swelling of the liver and exudation of fluid from the surface. Replacement of albumin by 2·6% (w/v) dextran 40 or dextran 80 led to cessation of flow, mainly because of clumping of red cells (see Grönwall, 1957). Varying the albumin concentration between 1·5 and 6% made no difference. Haemaccel deionized, 2·6% (w/v) (Behring Werke, Marburg, Germany), a 'plasma expander' (Schmidt-Thomé, Mager & Schöne, 1962) prepared from gelatin with a molecular weight about 35000, used instead of albumin gave good perfusion flow but some haemolysis occurred and the rate of glucose formation from lactate was about 15% lower [0·9 ± 0·1 (4) $\mu$mole/min./g.].

Omission of red cells made no difference provided that the livers weighed below 5g. and that the flow rate was rapid (not less than 30ml./min.). Addition of red cells equivalent to 2·5% haemoglobin provided a safety margin with respect to oxygenation; higher red cell concentrations were of no advantage. With 2·5% haemoglobin, flow rates as low as 10ml./min. were sufficient, as was oxygen in place of 95% oxygen.

Omission of calcium, or potassium, or phosphate, or magnesium decreased the rate of gluconeogenesis to about half the standard rate (Table 5). Replacement of the sodium salts by potassium salts caused an immediate block of the liver blood vessels. Replacing the 25mM-sodium bicarbonate–carbon dioxide buffer by 10mM-sodium phosphate buffer did not affect the rate of glucose synthesis. Owing to the continuous production of carbon dioxide and $HCO_3^-$ by the tissue the phosphate buffer medium was not free from bicarbonate. A concentration of 3–4mM-$HCO_3^-$ was maintained by monitoring pH of the medium and adjusting it from time to time with N-hydrochloric acid to pH 7·4.

The effect of pH and bicarbonate was further examined in a series of experiments in which pH was varied by changing the concentration of bicarbonate in the medium and the carbon dioxide content of the gas mixture. The bicarbonate concentration of the medium was measured in the Natelson micro-gasometer at approximately mid-time of the period during which gluconeogenesis was measured. The carbon dioxide content of the gas mixture was determined manometrically by the method of Gevers & Krebs (1966). The pH was calculated from the dissociation constant of carbonic acid, p$K$ being taken as 6·10. The checks by electrometric measurements in the reservoir of the oxygenator gave good agreement. During the perfusion with lactate the concentration of bicarbonate rose but the effect on pH was small during the critical period when the rate was measured. The rate of gluconeogenesis was about the same between pH 7·1 and 7·7 (Table 6) and within the range tested it was independent of the concentrations of bicarbonate and carbon dioxide. Beyond pH 7·7 the rate fell gradually and reached about half the maximal rate at pH 8·2. It fell very sharply at the acid side of pH 7·1, reaching about one-tenth of the maximal rate at pH 7·0. This sharp fall was confirmed in many experiments.

*Bile production.* In seven experiments bile was excreted at the rate of 67 ± 7mg./hr./g. wet wt. Bromosulphophthalein when added to the perfusion medium promptly appeared in the bile.

*Test for bacterial infection.* Staining and microscopic examination of the perfusion fluid after 130min. never revealed the presence of bacteria. Addition of Terramycin (4mg./150ml.), which does not affect the rate of gluconeogenesis and has been suggested by Schimassek (1963*a*) as a precautionary measure, was avoided because it is known to be an inhibitor of protein synthesis (Gale, 1963).

Table 5. *Effects of modifications of the perfusion medium on glucose synthesis from lactate*

Livers of rats starved for 48hr. were perfused with media from which one standard component was omitted. When bicarbonate was omitted it was replaced by 10mM-sodium phosphate buffer, pH 7·4, and the gas was $O_2$ without $CO_2$. L-Lactate was 10mM initially. For further details see the text. Three measurements were made for each condition.

| Perfusion medium | Rate of glucose synthesis ($\mu$moles/min./g.; mean ± S.E.M.) |
|---|---|
| Standard medium | 1·06 ± 0·09 |
| $Ca^{2+}$ omitted | 0·42 ± 0·18 |
| $Mg^{2+}$ omitted | 0·67 ± 0·23 |
| $K^+$ omitted | 0·36 ± 0·05 |
| Phosphate omitted | 0·53 ± 0·07 |
| $HCO_3^-$ omitted | 1·10 ± 0·13 |

Table 6. *Effect of* pH *on gluconeogenesis from lactate in the perfused rat liver*

Livers of rats starved for 48hr. were used. The pH was varied as described in the text. As a rule different livers were used for each pH value but in a few cases pH was altered during the experiments by addition of $NaHCO_3$ solution or by altering the $CO_2$ content of the gas mixture. For further details see the text.

| Concn. of $HCO_3^-$ (mM) | 9·0 | 11·8 | 15·0 | 9·0 | 35·0 | 25·0 | 15·0 | 39·0 | 35·0 | 32·0 | 40·0 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $CO_2$ content of gas mixture (%) | 5·0 | 5·0 | 5·0 | 2·5 | 7·0 | 5·0 | 2·5 | 5·0 | 2·5 | 1·4 | 1·4 |
| Concn. of $CO_2$ in medium (mM) | 1·12 | 1·12 | 1·12 | 0·56 | 1·57 | 1·12 | 0·56 | 1·12 | 0·56 | 0·31 | 0·31 |
| pH | 7·01 | 7·12 | 7·23 | 7·35 | 7·45 | 7·45 | 7·53 | 7·64 | 7·90 | 8·11 | 8·21 |
| Rate of gluconeogenesis (μmoles/min./g.) | 0·10 | 1·10 | 1·00 | 1·06 | 1·00 | 1·06 | 1·00 | 1·03 | 0·85 | 0·80 | 0·50 |

Table 7. *Adenine nucleotides in perfused rat liver*

Livers of rats starved for 48hr. were perfused with the standard medium. Lactate (1500μmoles) was added at 38min. For the analytical procedures see the Experimental section. The initial values refer to livers of rats starved for 48hr. and anaesthetized with ether; they are quoted from Underwood (1965). Results are given as means ± S.E.M.

| Substrate added to medium | Duration of perfusion (min.) | No. of observations | ATP (μmoles/g.) | ADP (μmole/g.) | AMP (μmole/g.) | Ratio $\frac{ATP}{ADP}$ | Total adenine nucleotides (μmoles/g.) |
|---|---|---|---|---|---|---|---|
| Nil | 5 | 3 | 2·18±0·10 | 0·89±0·06 | 0·37±0·03 | 2·46±0·20 | 3·44±0·12 |
| Nil | 15 | 3 | 2·17±0·15 | 0·86±0·03 | 0·36±0·02 | 2·54±0·10 | 3·39±0·16 |
| Nil | 85 | 3 | 2·05±0·15 | 0·66±0·01 | 0·44±0·03 | 3·11±0·18 | 3·15±0·12 |
| Lactate | 85 | 5 | 1·70±0·25 | 1·00±0·10 | 0·30±0·04 | 1·76±0·18 | 3·00±0·20 |
| Lactate | 130 | 4 | 1·70±0·08 | 0·86±0·02 | 0·26±0·04 | 1·99±0·07 | 2·82±0·12 |
| Initial values | 0 | 5 | 2·53±0·16 | 0·94±0·09 | 0·21±0·06 | 2·70±0·26 | 3·68±0·31 |

*Stability of adenine nucleotides in the perfused liver*

As Schimassek (1963*a*) has pointed out, the concentrations of the three adenine nucleotides are indicators of the physiological state of the perfused liver. In his experiments the anoxia during the preparation of the liver for the perfusion caused a fall in the concentration of ATP from 2·9 to 0·7mM and prolonged perfusion led to loss of total adenine nucleotide. The ATP lost initially was resynthesized within 30min. In the present experiments (Table 7) there was no significant change in the concentration of adenine nucleotides 5min. after the start of perfusion. Thus any changes during the preparation were promptly corrected. The total adenine nucleotide concentration fell about 15% after 85min. The change between 85 and 130min. was slight. Lactate caused a fall in the ATP/ADP ratio from 3·1 to 1·8, which can be explained by the increased rate of ATP consumption during gluconeogenesis from lactate. The initial values for total adenine nucleotides were a little lower than reported by Schimassek (1963*a*) and by Exton & Park (1965) but were within the range reported by other authors (see summary by Bücher, Krejci, Rüssmann, Schnitger & Wesemann, 1964).

## DISCUSSION

*Assessment of perfusion system.* The results obtained indicate that the method of perfusion is satisfactory for the study of gluconeogenesis from lactate inasmuch as the observed rates were rather higher than the maximal rates measured in the living animal. Hornbrook, Burch & Lowry (1965) found in rats starved for 24hr. a rate of about 0·5μmole of glucose equiv. formed/min./g. of liver on peritoneal administration of lactate. This rate is about half the average rate found in the present experiments and in the liver perfusions of Schimassek (1963*b*) and of Exton & Park (1965). The observed rate of 1μmole/min./g. means that 260mg. of glucose equiv. is synthesized/g. of tissue/day; thus, given sufficient lactate, the liver can form an amount of glucose equivalent to its own dry weight within 24hr. The rate of urea synthesis from ammonia under optimum conditions was even higher, and far exceeded those which can possibly be reached under physiological conditions where rates are limited by the quantity of substrate available.

A major advantage of the modified method is the short spell of interruption of circulation.

Schimassek (1963*a*) noted that after the 7–9min. period during which circulation was interrupted the concentrations of lactate in the liver rose eightfold, of α-glycerophosphate sixfold and that of ATP fell to one-quarter. Restoration of the initial state required almost 30min. An advantage of aged human red cells from a blood bank instead of rat blood is the cheapness, convenience and the elimination of blood glycolysis.

Energy supply did not appear to be a limiting factor since gluconeogenesis was not inhibited when addition of ammonia greatly increased the ATP requirements of the liver.

*Failure to deposit glycogen.* A special feature of the perfused starved liver was the fact that glucose was the sole major carbohydrate formed from lactate under the test conditions. No glycogen was deposited and the remnants of preformed glycogen were largely shed, presumably because of the low glucose concentration in the medium.

*Extra oxygen consumption induced by substrates.* The extra oxygen consumption caused by lactate and especially by ammonium chloride substantially exceeded that expected on the basis of the ATP requirements, a conclusion which would not be affected by the minor uncertainties of the efficiency of oxidative phosphorylation under the test conditions. It is probable that the oxygen consumption not accounted for arose from energy-consuming processes induced by the substrates. The observations by Schimke (1962*a*,*b*) that a high-protein diet stimulates the synthesis of urea-forming enzymes raises the question whether ammonia might directly or indirectly be an inducer of enzyme synthesis in the liver and whether the increased oxygen consumption is connected with an increased rate of protein synthesis.

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