A System for the Study *in situ* of the Pentose Phosphate Pathway in Rabbit Liver

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A surgical procedure for the isolation of the liver from the systemic circulation of the anaesthetized rabbit is described. The technique allowed the metabolism *in situ* of intraportally infused substrates to be followed for periods up to 5 min, free from the contaminating influences of metabolism by other body tissues. Details of the procedures necessary to achieve the uniform infusion, homogeneous distribution and containment of ^{14}C -labelled glucose substrates in the liver by haemostasis are described. Changes in $p\text{O}_2$, $p\text{CO}_2$, pH and the concentrations of NADP^+ , NADPH and glucose during each minute interval of the total 5 min period of metabolism are given. Reactant ratios of the lactate dehydrogenase system and the adenine nucleotide system have been calculated from the concentrations of the pertinent metabolites for the same period of metabolism. Glucose production by rabbit liver *in situ* proceeded at the rate of $1.08 \mu\text{mol}/\text{min}$ per g wet wt. of liver during the 5 min metabolic interval. The presence of the oxidative reactions of the pentose phosphate pathway of glucose metabolism was inferred from the quotient oxidation of $[1\text{-}^{14}\text{C}]\text{glucose}/\text{oxidation of } [6\text{-}^{14}\text{C}]\text{glucose} = 1.8$.

Muntz & Murphy (1957) described a procedure for the investigation of pathways of glucose metabolism in rat liver *in vivo*. Their method involved the injection of specifically labelled $[^{14}\text{C}]\text{glucose}$ into the hepatic portal vein of the anaesthetized rat. The presence of specific pathways of carbohydrate metabolism was inferred from the degree of isotope incorporation into CO_2 and specific carbon atoms of lactate and hexose monophosphate isolated from the liver after metabolism. The technique of Muntz & Murphy (1957) has been applied more recently in an investigation of the metabolism of fructose in liver *in vivo* (Muntz & Vanko, 1962; Muntz, 1968). These experiments indicated the presence of a significant pathway of fructose metabolism in liver which is distinct from the fructose 1-phosphate aldolase (EC 4.1.2.7) pathway (Hers & Kusaka, 1953), which had been demonstrated by using liver homogenate.

The technique of Muntz & Murphy (1957) has limitations that do not permit its use for more extensive investigations, such as those involving kinetic examination of the mechanism of metabolic pathways and the measurement of their quantitative contribution to metabolism. These restrictions are due to the very short time (usually 90 s) that is allowed for the labelled substrate to be

metabolized in the liver. The use of short exposure times is a necessary condition of the technique to prevent the re-entry of ^{14}C -labelled intermediates of metabolism into the portal vein after their exit from the hepatic veins and passage through peripheral tissues.

This paper is concerned with a technique by which the intact rabbit liver can be used to investigate the occurrence, reaction sequence and quantitative extent of pathways of carbohydrate metabolism *in situ*. The technique has the advantage of maintaining the organization and physiological function of the intact liver while isolating the organ from the systemic circulation for a brief period. The technique allows the full metabolic activity of the entire mass of the organ to be utilized experimentally and has the advantage of permitting the metabolic transformation, in short time-intervals, of sufficient quantities of substrate to allow the subsequent isolation and analytical characterization of intermediary products. It has been used extensively in investigations of the mechanism of the pentose phosphate cycle *in situ* (see the following paper, Williams, Rienits, Schofield & Clark, 1971, and also Clark, 1967). Some physiological and biochemical properties of the liver *in situ* are presented as part of the assessment of the system.

MATERIALS AND METHODS

Animals. California–New Zealand White cross-bred female rabbits, 16–20 weeks of age, were obtained from the Tillside Stud, Bargo, N.S.W., Australia. In all the investigations on the system the rabbits were starved by depriving them of all food but not water for 40 h before their use in experiments.

Radiochemicals. D-[1- 14 C]- and D-[6- 14 C]-glucose were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Reagents. Glucose oxidase (EC 1.1.3.4) and Florisil (60–100 mesh) were supplied by Sigma Chemical Co. (St Louis, Mo., U.S.A.), *o*-dianisidine and NaOH were obtained from British Drug Houses (Poole, Dorset, U.K.), HClO_4 and BaCl_2 from May and Baker Ltd. (Dagenham, Essex, U.K.) and Evans Blue (Azovan Blue) from George T. Gurr Ltd. (London S.W.6, U.K.).

All enzymes and coenzymes used for the determination of metabolites were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K.

Radioactivity. The radioactivity of $\text{Ba}^{14}\text{CO}_3$ was determined by using a windowless automatic gas-flow counter (Nuclear–Chicago model D47/C110B). Sufficient counts were recorded in both sample and background to yield in the net count rate a standard error of less than 5% (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). All counts were corrected to infinite thinness.

Preparation of liver system and infusion procedure. The animal was anaesthetized with an ether–Carbogen ($\text{CO}_2 + \text{O}_2$, 5:95) gas mixture, and placed in a supine position on a warm operating table and secured by the limbs. After shaving the abdominal area, a mid-line incision was made from the xiphoid cartilage to the pubis, and the stomach and intestines were retracted to expose the porta hepatis. A syringe needle, connected to a motor-driven perfusor (Braun Perfusor model 1850) containing the solution of radioactive substrate was placed in the hepatic portal vein. The falciform ligament securing the junction of the right and left segments of the median liver lobe to the mid region of the diaphragm was severed.

The incision was extended laterally below the costal margin to mid-flank, thus exposing the hepatocaval junction. By hand the liver was retracted away from the diaphragm and the jaws of a pair of angled intestinal clamps (Doyen 9 in clamps) were placed at the hepatocaval junction. The liver was then infused with substrate (radioactive glucose in the experiments described in this paper), at a known rate for a short time-interval. Immediately after the infusion was complete, the flow of blood from the hepatic veins into the posterior vena cava was blocked by tightening the jaws of the clamps. The system, with infused substrate retained in the liver, was left for measured time-intervals (hereafter referred to as the 'holding time'). During the holding time the flow of ether was stopped but the Carbogen gas flow was continued. The animal remained in an anaesthetized state exhibiting normal respiration and heart rate during this period.

At the end of the holding time the whole liver was removed as rapidly as possible (5 s) into a stainless-steel mortar (1 litre volume) filled with liquid N_2 and quickly broken up to form a deep-frozen homogeneous fine powder from which appropriate metabolites could be extracted (Williams *et al.* 1971).

Isolation of carbon dioxide and determination of specific radioactivity. Carbon dioxide was isolated from a 10 g portion of frozen liver powder in an apparatus designed for the distribution and isolation of gases (Fig. 1).

CO_2 was liberated from liver by treatment with 0.6 M- HClO_4 , added to flask A from funnel B, while the entire manifold system was maintained at a pressure not exceeding 0.5 mmHg. CO_2 was trapped in the condenser E, which was immersed in liquid N_2 . The release and collection of CO_2 were complete in 20 min. The condenser containing the gas was closed, removed from the manifold and the CO_2 distilled into 1 M-NaOH contained in the centre well of a modified Warburg flask. The trapped CO_2 (as Na_2CO_3) was converted into BaCO_3 and the specific radioactivity determined.

Oxygen concentration, $p\text{CO}_2$ and pH of hepatic blood during the holding time. For this determination the liver was exposed by a ventral mid-line incision and extended with a left lateral transverse incision sufficient to expose and allow ligation of the posterior vena cava caudal to the confluence of the hepatic veins and the vena cava. A clamp was positioned distal to the hepatocaval junction. The distance between the ligation and the clamp was not greater than 0.5 cm. Hepatic venous blood was anaerobically sampled as follows. A needle, which had been flushed with N_2 and with the barrel adaptor sealed, was inserted into the largest of the hepatic veins. The seal of the needle adaptor could be quickly removed to allow the attachment of a N_2 -flushed well-greased syringe. Samples of blood (approx. 1 ml) were sequentially taken from the liver. The syringes were sealed immediately and placed in an ice bath. The $p\text{O}_2$, $p\text{CO}_2$ and pH were determined within 15 min of sampling by means of a pH and Blood Gas Analyser (model 113; Instrumentation Lab. Inc., Boston 72, Mass., U.S.A.).

Microscopic examination of liver. The technique of clamping the hepatic veins caused the liver veins and sinusoids to become engorged with blood. To examine the visible effect of short periods of venous engorgement on cells, the liver was clamped to establish haemostasis for intervals ranging from 1 to 5 min, the portal vein and hepatic artery were then ligated and the whole liver was excised and immersed in liquid N_2 . By using the freezing microtome, thin (5–7 μm) sections were cut, stained with haematoxylin and eosin (Drury & Wallington, 1967) and examined with the light microscope. Normal non-engorged liver sections were similarly prepared and served as controls.

Blood content of extirpated liver. An estimate of the volume of blood contained in the extirpated liver was obtained by an indirect method. A direct determination of haemoglobin in the liver powder (and hence blood volume) was not feasible since the cytochromes of liver would interfere with the spectrophotometric assay for haemoglobin. The procedure adopted was as follows. The liver of the anaesthetized rabbit was exposed and the circulation terminated by clamping. The portal and hepatic veins as well as the bile duct were cannulated. The blood-vessel cannulae were connected to a perfusion apparatus and the whole liver was perfused with 250 ml of 0.9% NaCl (Gordon, 1968). The perfusion was allowed to proceed for 20 min, by which time the blood contained in the liver was thoroughly mixed with the perfusion fluid. A standard curve of E_{540} of known dilutions of hepatic

Fig. 1. Apparatus for the liberation and isolation of CO_2 from liver. A, Flask containing powdered liver; B, funnel for the addition of HClO_4 ; C, McLeod vacuum gauge; D, water-vapour trap in an ice-salt bath (-20°C); E, condenser for trapping CO_2 ; F, manometer vacuum gauge; G, vapour trap in liquid N_2 ; H, mercury diffusion pump. Other details are given in the Materials and Methods section.

venous blood was prepared. The amount of blood in the saline perfusate was determined spectrophotometrically by reference to the standard curve.

Determination of metabolites. Approx. 20 g of the frozen pulverized liver was extracted with HClO_4 and neutralized with KOH as described by Williamson, Lund & Krebs (1967). Lactate and pyruvate were determined by the method of Hohorst, Kreutz & Bücher (1959). ATP was determined by the method of Lamprecht & Trautschold (1963) and ADP and AMP by the method of Adam (1963). The extracts were shaken with Florisil, as described by Williamson *et al.* (1967), to eliminate the slow non-enzymic oxidation of NADH observed with untreated samples. As Florisil interfered with the recovery of adenine nucleotides it was not employed for the extracts used for the determination of these substances. P_i was determined by the method of Martin & Doty (1949).

A separate sample of frozen liver powder was extracted by the method of Burch, Lowry & Von Dippe (1963) for the determination of NADP^+ and NADPH. These nucleotides were determined by the methods of Klingenberg (1963).

Glucose formation. Glucose released into the blood of the liver was measured by using the glucose oxidase method (Huggett, 1957). Blood was sampled from the liver by using the same surgical and sampling techniques described for the determination of the $p\text{O}_2$ of hepatic venous blood.

RESULTS

Effect of infusion time on $^{14}\text{CO}_2$ production. It is apparent that the extent of metabolism of an infused substrate would be influenced by the quantity remaining within the liver during the holding time and its distribution within the organ. Infusion conditions giving rise to maximum distribution throughout the liver as well as maximum containment of substrate would presumably give rise to maximum rates of metabolism of the infused substrate during the holding time.

A series of determinations were made in which $[1-^{14}\text{C}]$ glucose served as substrate and the specific radioactivity of CO_2 present in the liver at the end of a 5 min holding time was used as an index of metabolism. The infusion volume was kept constant at 1.0 ml and contained $10\mu\text{Ci}$ and 0.06 mg of glucose. The time of infusion was varied from 5 to 30 s, i.e. the infusion rate varied from 2.0 to 12.0 ml/min. As seen from the results of Fig. 2 an infusion time of 15 s achieved a maximum specific radioactivity of liver CO_2 . This result is in agreement with the theoretical time required to completely discharge and replenish the blood in rabbit

liver. The theoretical value was estimated from the hepatic portal blood flow rate (100 ± 5.7 ml/min) (White, Chalmers, Hilder & Korner, 1967) and the vascular volume of the liver (17.8–21.7%) (Brauer, 1956).

The distribution of the infusion medium was visually confirmed by the infusion of 1 ml of 0.5% Evans Blue solution (*U.S. Pharmacopeia*, 1960) into the portal vein of the anaesthetized rabbit at a rate of 4 ml/min. Post-mortem examination of liver showed that an even distribution of the dye through and up to the peripheral edge of all liver lobes had occurred.

[1- 14 C]Glucose served as the substrate of choice in determining the optimum period of infusion since it produced $^{14}\text{CO}_2$ much more rapidly than any other specifically labelled glucose. Variation of the nutritional state of the rabbit, ranging from fed to 100h-starved, did not significantly alter the relationship between the duration of infusion and the specific radioactivity of CO_2 produced. As a result of the experiment described above and a number of others in which various amounts of [1- 14 C]-glucose were tried, $10 \mu\text{Ci}$ of specifically labelled sugar was administered in 1 ml of solution infused over 15s in all experiments.

C-1/C-6 ratio of rabbit liver *in situ*. The specific radioactivities of CO_2 after the metabolism of [1- 14 C]- and [6- 14 C]-glucose are shown in Table 1. The results indicate good agreement between individual animals. A value of 1.8 was obtained for the C-1/C-6 ratio (the ratio of specific radioactivity of CO_2 arising from [1- 14 C]glucose to that from

[6- 14 C]glucose). Previous determinations were 1.28 for starved liver *in vitro* (Agranoff, Brady & Colodzin, 1954) and 2.5 for fed rabbit liver (Bloom, 1955). The magnitude of the ratio *in situ* confirmed the existence of the oxidative reactions of the pentose phosphate pathway for glucose metabolism in the liver of the rabbit (Abraham, Cady & Chaikoff, 1957).

Contamination of the excised liver preparation with blood. The surgical removal of the whole rabbit liver into liquid N_2 necessarily involved some contamination of the liver homogenate with blood. Erythrocytes (Brin & Yonemoto, 1958; Murphy, 1960) and leucocytes (Beck, 1958) are both reported to utilize the pentose phosphate pathway for glucose metabolism. Since the technique and procedures described were developed primarily for the investigation of the pentose phosphate pathway in liver, it was necessary to estimate the effect of blood-cell contamination on the metabolism of glucose by liver.

The rabbits had a liver weight of 82.56 ± 14.61 (14) g and were found to contain 4.37 ± 0.54 (3) ml of blood. This is considerably less than the estimated blood volume of liver (17.8–21.7%) (Brauer, 1956). The low estimate of 4.37 ml of whole blood in liver is explained by the finding of Brauer (1956) that liver parenchymal cells are bathed by a medium in relatively rapid equilibrium with the blood plasma passing through the liver, whereas erythrocytes are confined to a space occupying only 10% of the parenchyma.

Calculations based on the data of Brin & Yonemoto (1958), Murphy (1960) and Rapoport (1968) indicated that the erythrocytes present in 4.4 ml of blood would contribute less than 1% of the radioactivity found in CO_2 from the liver preparation when either [1- 14 C]- or [6- 14 C]-glucose was

Fig. 2. Effect of infusion time of [1- 14 C]glucose on the specific radioactivity of $^{14}\text{CO}_2$ from liver. The [1- 14 C]glucose was infused intraportally into the rabbit liver. The vertical lines indicate $\pm$ S.D. Experimental conditions are given in the text.

Table 1. Specific radioactivity of CO_2 resulting from the metabolism of [1- 14 C]- and [6- 14 C]-glucose in rabbit liver *in situ*

A 1.0 ml sample of 0.33 mM-glucose containing $10 \mu\text{Ci}$ of specifically ^{14}C -labelled glucose was infused for 15s into the portal vein of the anaesthetized rabbit at the rate of 4.0 ml/min. The labelled glucose was confined in the liver for 5 min by haemostasis. CO_2 was isolated from liver and the radioactivity determined as described in the Materials and Methods section. The values are the means $\pm$ S.D. with numbers of observations in parentheses.

	$10^{-3} \times \text{Sp. radioactivity of } \text{CO}_2$ (c.p.m./mmol)
[1- 14 C]Glucose	20.24 ± 3.4 (13)
[6- 14 C]Glucose	11.30 ± 2.9 (10)
C-1/C-6 = 1.8	

used as substrate. The contribution to radioactivity in CO_2 due to leucocytes and platelets present in the blood calculated from the data of Stjernholm (1967) and Cline (1965) would be even less. Results obtained in this laboratory by Gordon (1968) indicated that the contribution to radioactivity in CO_2 from rabbit blood was 0.3% with $[1\text{-}^{14}\text{C}]\text{-glucose}$ as the substrate. Therefore it is concluded that contamination by blood is of no real significance in assessing the data obtained on the liver preparation.

Oxygen concentration in liver cells during the holding time. Obstruction of the draining hepatic veins by the experimental technique resulted in a progressive hepatic ischaemia and necessitated the investigation of this effect on normal oxidative metabolism during the period of observation.

The results in Table 2 show the changes in the concentration of oxygen in the blood sampled from the hepatic veins at various intervals during the holding time.

Effect of progressive ischaemia on the reactant ratios of the lactate dehydrogenase system and the adenine nucleotide system in rabbit liver. The concentration of lactate, pyruvate, P_i and adenine nucleotides in rabbit liver and their changes during a 5 min interval of the holding time are shown in Table 3. There were no appreciable changes in the tissue concentrations of lactate or AMP up to 5 min after obstruction of the hepatic veins. The concentration of pyruvate decreased considerably so that the $[\text{lactate}]/[\text{pyruvate}]$ ratio increased. The cytoplasmic $[\text{free NAD}^+]/[\text{free NADH}]$ ratio, as cal-

culated from the $[\text{lactate}]/[\text{pyruvate}]$ ratio (Williamson *et al.* 1967) also decreased. The concentrations of ATP and ADP both changed; ATP decreased markedly from 1.21 to 0.39 and ADP decreased from 0.91 to 0.65, so that whereas $[\text{HPO}_4^{2-}]$ increased only slightly, the $[\text{ATP}]/[\text{ADP}][\text{HPO}_4^{2-}]$ ratio decreased substantially. The marked decrease in the concentration of ATP also influenced the value of the reactant ratio of the adenylate kinase system which was noted to decrease from 1.14 to 0.68 during the 5 min holding time.

pH and $p\text{CO}_2$ changes during holding time. The results in Table 2 show that the pH values of blood were normal (Castelman & Kibbee, 1963) during a 7 min period of ischaemia. The $p\text{CO}_2$ values gradually increased, consistent with the accumulation of CO_2 from substrate oxidations during the holding time.

Changes in NADP^+ and NADPH concentrations during holding time. Fig. 3 shows the changing concentrations of NADP^+ and NADPH during 15 min ischaemia. The concentration of NADP^+ decreased by approx. one-third and this was accompanied by an equivalent increase in NADPH .

Glucose production during holding time. Glucose production by the liver proceeded unimpaired during the holding time. From the results in Fig. 4 it was calculated that glucose was released into the blood at the rate of 16.0 ± 5.2 (7) mg/min per 82.6 g wet wt. of liver during the 5 min of arrested hepatic circulation. This is equivalent to the rate of gluconeogenesis from lactate in the isolated perfused rat liver (Exton & Park, 1965; Hems, Ross, Berry & Krebs, 1966). The amount of glucose formed in the liver was calculated from the rate of increase in hepatic blood glucose, the vascular volume of liver and the average weight of rabbit liver.

Microscopic appearance of liver. The light-microscopic appearance of cells from livers which were engorged for periods of 1–5 min was not distinguishable from the microscopic appearance of normal liver cells. This indicated that venous engorgement for short intervals of time had not imparted any visible disfigurement to the cellular structures.

Table 2. *Changes in gas content and pH of blood sampled from liver at various intervals during holding time*

The total amount of oxygen present in the blood was calculated from the $p\text{O}_2$ values and the oxygen dissociation data for rabbit blood (Korner & Darian-Smith, 1954). The techniques used for the sampling of blood from the hepatic veins and for the measurement of $p\text{O}_2$, $p\text{CO}_2$ and pH values are recorded in Materials and Methods section. Results are mean values and the numbers of observations are shown in parentheses.

Time (min)	$p\text{O}_2$ (mmHg)	Total O_2 (ml/100 ml)	$p\text{CO}_2$	pH
0	50.0 (5)	18.38	44.0 (3)	7.38 (3)
1	35.5 (1)	13.62	54.0 (3)	7.41 (3)
2	28.5 (3)	9.74	52.0 (3)	7.46 (3)
3	22.0 (1)	7.57	58.0 (3)	7.50 (3)
4	20.0 (5)	6.06	58.0 (3)	7.45 (3)
5	18.5 (5)	5.42	63.0 (3)	7.49 (3)
6			62.0 (3)	7.46 (3)
7			62.0 (3)	7.48 (3)
10	12.5 (5)	2.82		
15	6.5 (5)	1.41		

DISCUSSION

The liver system *in situ* provides experimental material for the study of a number of metabolic processes in liver. It possesses certain advantages over the fully perfused liver preparation *in vitro*, in that complex apparatus is not necessary and the organ is retained within the animal during the 'incubation'. The problem of cation losses associated with tissue slices (Flink, Hastings & Lowry, 1950) is considerably decreased with the intact liver (Mortimore, 1961). The diffusion of nutrients

Table 3. *Concentrations of metabolites, reactant ratios of the lactate dehydrogenase system and the adenine nucleotide system in rabbit liver in situ during holding time*

Starved rabbits (female, 3.0–3.5 kg) were anaesthetized (15 min) and the livers surgically exposed. An ischaemic condition was imposed on the liver by clamping shut the outflow of blood from the hepatic veins for the times shown. NaCl (0.9%, 1.0 ml) was infused into the liver intraportally at the rate of 4.0 ml/min for 15 s before closing the hepatic veins. The results are mean values ($\pm$ s.d.) for five animals. The concentration of HPO_4^{2-} at pH 7.0 was taken to be 60% of the total P_i , and the values for ADP have been corrected for the presence of other nucleotides in a manner similar to that reported by Goldberg, Passonneau & Lowry (1966). The method of the calculation of the $[\text{free NAD}^+]/[\text{free NADH}]$ ratio was described by Williamson *et al.* (1967). For other details see the Materials and Methods section.

Metabolite	Concn. of metabolite ($\mu\text{mol/g}$ fresh wt.)					
	0 min	1 min	2 min	3 min	4 min	5 min
Lactate	4.54 $\pm$ 0.97	5.22 $\pm$ 1.33	4.17 $\pm$ 1.38	6.76 $\pm$ 1.40	4.38 $\pm$ 1.72	5.82 $\pm$ 1.83
Pyruvate	0.122 $\pm$ 0.041	0.139 $\pm$ 0.044	0.115 $\pm$ 0.032	0.164 $\pm$ 0.035	0.075 $\pm$ 0.032	0.086 $\pm$ 0.022
ATP	1.21 $\pm$ 0.16	1.21 $\pm$ 0.18	0.81 $\pm$ 0.09	0.62 $\pm$ 0.09	0.59 $\pm$ 0.11	0.39 $\pm$ 0.13
ADP	0.91 $\pm$ 0.08	0.98 $\pm$ 0.07	0.92 $\pm$ 0.07	0.76 $\pm$ 0.08	0.62 $\pm$ 0.11	0.65 $\pm$ 0.09
AMP	0.78 $\pm$ 0.15	0.98 $\pm$ 0.28	1.05 $\pm$ 0.25	0.57 $\pm$ 0.19	0.63 $\pm$ 0.20	0.74 $\pm$ 0.25
P_i	4.79 $\pm$ 0.14	4.63 $\pm$ 0.12	5.94 $\pm$ 0.15	5.17 $\pm$ 0.13	6.43 $\pm$ 0.12	5.61 $\pm$ 0.11
[Lactate]/[pyruvate]	37.21	37.55	36.26	41.22	58.40	67.67
$10^3 \times [\text{ATP}]/[\text{ADP}][\text{HPO}_4^{2-}]$	463	444	247	263	247	178
$[\text{ATP}][\text{AMP}]/[\text{ADP}]^2$	1.14	1.23	1.00	0.61	0.97	0.68
[Free NAD^+]/[free NADH]	242	240	248	219	154	133

Fig. 3. Effect of holding time on the concentrations of NADP^+ (●) and NADPH (○) in rabbit liver. The vertical lines indicate $\pm$ s.d. Experimental conditions are given in the text.

Fig. 4. Effect of holding time on the rate of glucose production in rabbit liver *in situ*, as measured by the accumulation of glucose in hepatic venous blood. The vertical lines indicate $\pm$ s.d. Experimental conditions are given in the text.

into liver cells is facilitated because the supply of substrates takes place through capillary circulation. Since there is a minimum of handling a greater quantity of undamaged tissue is available that may be of some importance when metabolic intermediates or enzyme activities are to be measured (Newsholme & Gevers, 1967).

The liver, however, is not in its situation *in vivo* because it is isolated during the brief holding time and whereas this isolation enabled metabolism to be examined free from the contributions of other

tissues, it does mean that the system under study is not completely physiological. However, the system retained the structural integrity of the liver and therefore could be expected to provide both quantitative and qualitative information on metabolic events *in vivo* more directly than slices or homogenates.

From a consideration of the results shown in Tables 2 and 3 it is apparent that during the 5 min holding time metabolism in the liver has become partially deranged. The concentration of pyruvate decreased and although the concentration of lactate

remained constant there was an overall increase in the [lactate]/[pyruvate] ratio and a twofold decrease in the [free NAD⁺]/[free NADH] ratio. These results contrasted with those reported for the totally ischaemic rat liver studied for the same interval (Brosnan, Krebs & Williamson, 1970), where increased concentrations of lactate contributed in part to the observed increase in the [lactate]/[pyruvate] ratio. In general these results would be consistent with a decrease in the availability of oxygen (as shown in Table 2 the content of O₂ in hepatic blood decreased from 18.38 to 5.42 ml/100 ml during the 5 min holding period). However, the observed change in the [lactate]/[pyruvate] ratio, together with the decrease in the [ATP]/[ADP][HPO₄²⁻] ratio may have also arisen in part from the undiminished rate of gluconeogenesis, inferred from the continuous rate of glucose production (Fig. 4) and the failure to demonstrate the presence of glycogen in the liver of the 40 h-starved rabbit by using the method of Illingworth, Larner & Cori (1952). In a situation such as this where the rate of gluconeogenesis is high and presumably the requirements for ATP are high, a decrease in the phosphorylation state of the adenine nucleotide system may be followed by a change of the [NAD⁺]/[NADH] ratio on account of equilibrium relations between the cytoplasmic adenine nucleotides and nicotinamide nucleotides (Veech, Raijman & Krebs, 1970).

The relative constancy of the [free NAD⁺]/[free NADH] ratio, which falls by half during the 5 min holding time, in contrast with the eightfold fall in the ratio for the totally ischaemic situation (Brosnan *et al.* 1970), is not unexpected since the hepatic artery is not obstructed in this experimental system and collateral circulation is rapidly established in liver (Fischer, 1963).

Table 2 shows that the rate of loss of oxygen from the blood diminished markedly after the 5 min holding period. This may indicate that factors other than oxygen concentrations become important after approx. 5 min and may influence oxygen consumption. For this reason and the observed increased [lactate]/[pyruvate] ratio at greater than 5 min holding times (J. F. Williams, unpublished observations) the application of this technique to studies on carbohydrate metabolism have not been extended beyond 5 min.

Fig. 3 shows that the [NADP⁺]/[NADPH] ratio decreased by approximately one-third during the first 5 min holding period yet over three-quarters of the original concentration of NADP⁺ was available at 5 min, indicating that the reactions of the oxidative segment of the pentose phosphate pathway would continue unimpaired.

Further evidence of the usefulness of this procedure for the study of liver metabolism is provided

in the following paper (Williams *et al.* 1971), which together with the results of Clark (1967) indicate that the liver treated in the manner described was able to provide meaningful information on the mechanism and contribution of the pentose phosphate cycle of glucose metabolism.

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