Lipid Metabolism in the Perfused Chicken Liver

LIPOGENESIS FROM GLUCOSE, ACETATE AND PALMITATE

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A procedure for the perfusion of the isolated chicken liver was developed. The preparation satisfied many of the criteria of normal physiological function, e.g. oxygen consumption and the absence in the perfusion medium of enzymes indicative of cell damage, and retained its capacity to synthesize lipids from glucose, acetate and long-chain fatty acids. Part of newly synthesized triglyceride was released into the perfusion medium as lipoprotein.

The absorption, transport and synthesis of fat in birds shows important differences from that of non-avian species. Although fat digestion is apparently similar to that of mammals, in the absence of an active intestinal lymphatic system absorbed fat is carried directly to the liver in portal blood in the form of low-density lipoprotein (Noyan, Lossow, Brot & Chaikoff, 1964). In non-avian species examined to date adipose tissue is a major site of fat synthesis, but in the bird lipogenesis occurs mainly in the liver (Leveille, O'Hea & Chakrabarty, 1968). The key role of the liver in fat metabolism led us to develop a perfused liver preparation for the study of hepatic lipogenesis from glucose, acetate and free fatty acids. In all perfusion studies the maintenance of normal physiological function is the paramount requirement. Acceptable criteria of the efficiency of the perfused liver include the microscopic and histological appearance, oxygen consumption, rate of bile production, the overall synthetic capacity and the absence in the perfusate of enzymes indicative of cell damage, e.g. glutamate-pyruvate transaminase (L-alanine-2-oxoglutarate transaminase, EC 2.6.1.2).

MATERIALS AND METHODS

Analytical methods. Glucose was determined by the glucose oxidase method (Huggett & Nixon, 1957). The specific radioactivity of glucose in the perfusate was measured by adding carrier glucose (100 mg) to 3 ml of perfusate, deproteinizing with $\text{Ba}(\text{OH})_2\text{-ZnSO}_4$ and removing lactate and other ionic material from the supernatant by passage down a column of mixed-bed ion-exchange resins [British Drug Houses Ltd. (Poole, Dorset, U.K.); IRC50-IR45 (2:1, w/w)]. After concentration of the eluate *in vacuo*, glucose was isolated as the pentaacetate (Jones, 1965), and assayed for radioactivity by

scintillation counting in toluene (15 ml) containing 2,5-diphenyloxazole (4 g/l). Glycogen was isolated from liver by the method of Good, Kramer & Somogyi (1933). After hydrolysis with 1 M-HCl at 100°C for 6 h, the concentration and specific radioactivity of the glucose were measured as described above.

Acetate concentrations in the perfusion medium were measured by adding a known amount of a 'marker' fatty acid (isovalerate; 3-methylbutanoate), and isolating the volatile fatty acids by steam-distillation. The acetate concentration was calculated from the relative amount of acetate and isovalerate in the mixture, as determined by g.l.c. Sodium isovalerate (100 µg) was added to a sample of perfusate (5 ml), which was diluted with water (20 ml) and deproteinized with metaphosphoric acid (25%, w/v; 2 ml). The neutralized filtrate was concentrated *in vacuo* to 3–5 ml, the pH was adjusted to pH 2.0–3.0 with 5 M- H_2SO_4 (Bromophenol Blue indicator) and the solution was distilled in a Markham apparatus. The distillate (100 ml) was neutralized with 10 mM-NaOH under CO_2 -free conditions (phenolphthalein indicator), 50% excess of 10 mM-NaOH was added and the solution was concentrated to 5 ml by boiling. A portion (2 ml) was dried down in a radioactivity-counting vial under a stream of N_2 , dissolved in water (1 ml) and assayed for radioactivity by scintillation counting after the addition of 15 ml of toluene-Triton X-100 (2:1, v/v) containing 2,5-diphenyloxazole (4 g/l). The remainder of the fatty acid solution was concentrated and dried down in a small tube (3.0 cm × 0.5 cm), and the residue was dissolved in 50 µl of phosphoric acid (5%, v/v). The solution (3 µl) was injected directly into a Pye model 104 gas chromatograph with a stationary phase of neopentyl (2,2-dimethyl-prop-1-yl) glycol adipate (20%) on Celite (60–72 mesh, washed with phosphoric acid), with a flame ionization detector and an operating temperature of 125°C.

Haemoglobin concentrations in the perfusion medium were determined by measuring the extinction at 540 nm after dilution (1:100) with water. Standard curves relating haemoglobin concentration to E_{540} were constructed from solutions of known haemoglobin content, determined from the oxygen-binding capacity of the solutions (Peters & Van Slyke, 1932).

The concentration and specific radioactivity of free fatty acids and triglycerides in the perfusate and in liver tissue were determined by using internal markers (heptadecanoic acid and glycerol triheptadecanoate) as described by Bickerstaffe & Annison (1970).

Lactate concentrations in the perfusate were determined with lactate dehydrogenase (Hohorst, 1963). The specific radioactivity of the lactate was determined by the isolation of the zinc salt, after the addition of unlabelled carrier lithium lactate (52.4 mg), as described by Annison, Lindsay & White (1963). The crystalline product was assayed for radioactivity as a suspension in toluene containing low-density silicic acid [Cab-O-Sil; Packard Instrument Co., La Grange, Ill., U.S.A. (3–4 g/100 ml)] and 2,5-diphenyloxazole (4 g/l).

Glutamate-pyruvate transaminase was assayed by the u.v.-absorption method described by Wróblewski & Ladue (1956).

Radioactive materials. [^{14}C]Glucose (309 mCi/mmol), [^{14}C]acetate (29 mCi/mmol), [^{14}C]lactate (50 mCi/mmol) and [^{3}H]palmitate (350 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Perfusion apparatus. The apparatus, and the cabinet in which it was housed, was based on that of Hems, Ross, Berry & Krebs (1966). The temperature of the cabinet and of the circulating medium was maintained at 40°C. Modifications were made to the glassware to allow measurement of the rate of perfusate outflow by a simple graduated vessel, which could be isolated with a clamp to allow collection of perfusate for a known time (usually 30 s). The total volume of the perfusate was 300 ml. The pumping system and oxygenator were similar to those of Hems *et al.* (1966). A $\text{O}_2 + \text{CO}_2$ (95:5) gas mixture (Air Products, New Malden, Surrey, U.K.) was introduced into the oxygenator through a humidifier at 5 litres/min.

Perfusion technique. The medium of Hems *et al.* (1966), based on bovine albumin and aged washed human erythrocytes, proved unsatisfactory for the chicken liver preparation. Considerable haemolysis and clumping of the erythrocytes was observed. A satisfactory medium consisted of defibrinated chicken blood diluted (1:1, v/v) with Krebs–Ringer bicarbonate (Krebs & Henseleit, 1932) and containing haemoglobin at approx. 5.0 g/100 ml. The oxygen-carrying capacity of the diluted blood was adequate for the perfused preparation, as shown by the oxygen content of the perfusate after passage through the liver. Dilution of the blood decreased the amounts of endogenous substrates added to the system and minimized the glycolysis caused by the red cells. The blood was obtained from donor birds (male Cobb broilers, 18–20 weeks old), defibrinated, filtered through muslin and stored at 4°C for 48 h before use. During storage, the glycolytic activity of the nucleated erythrocytes declined to 5–10% of the initial value. To overcome possible limitations on lipogenesis imposed by shortage of amino acids for lipoprotein synthesis in the liver, the medium was supplemented with mixed amino acids (10 mg/100 ml) obtained from The Stuart Co., Pasadena, Calif., U.S.A. The composition of the perfusate (mg/100 ml) was: cystine+cysteine, 0.17; methionine, 2.9; aspartic acid, 6.9; threonine, 4.3; serine, 4.3; glutamic acid, 17.4; glycine, 2.1; alanine, 3.3; valine, 5.2; isoleucine, 4.1;

leucine, 9.3; tyrosine, 0.53; phenylalanine, 3.9; lysine, 7.5; histidine, 2.8; arginine, 3.5. Proline, ornithine and tryptophan were not measured.

The bird (male Cobb broiler, 8–10 weeks old) was anaesthetised by the injection of Nembutal (3.0 mg/100 g body wt.) into the wing vein and the abdomen was opened through a transverse incision made just below the point of the sternum. The incision was extended to right and left of the midline to reach to the caudal angles of the sternum. The ribs were cut away from the thoracic vertebrae on each side, with care being taken to avoid damaging the liver lobes which often adhere to the thoracic wall. Bone forceps were used to cut through the coracoid-clavicular articulation, and after division of the pectoral and supracoracoid muscles, the sternum was removed to expose the cardio-abdominal chamber.

The portal vein was isolated from the viscera and catheterized with a 25.0 cm length of Portex (Portland Plastics, Hythe, Kent, U.K.) polythene tubing (size 200), and immediately connected to a portable reservoir containing about 50 ml of the perfusion medium at 40°C, which was allowed to flush through the liver for about 5.0 s.

The inferior mesenteric and pancreatic veins were then tied off. The inferior vena cava, which passes through the right lobe of the liver and is located on the dorsal aspect of the abdominal cavity, is fully enclosed by the capsule of the liver and is also dorsal to the right suprarenal body. To avoid possible damage to the liver tissue by tearing the capsule, the inferior vena cava was clamped with artery forceps, and not tied off. The right and left cranial venae cavae were next tied off, and the apex of the ventricles was picked up with tissue forceps, so that the heart could be raised and held forward, while the pericardium was divided at the point where the hepatic veins enter the right auricle. Two loose ligatures were placed around the hepatic veins at this position.

The catheter, which was a length (25 cm) of transparent vinyl tubing, size NT/6 (Portland Plastics) was passed through a small incision made in the wall of the right auricle and its end was aligned into a position where both veins enter the atrium. The two loose ligatures were then pulled slightly forward to lie under the auricle and then tied to secure the catheter in place. Perfusion fluid from the reservoir was allowed to pass into the portal vein again, and its outflow from the hepatic catheter was checked, to ensure a leak-proof and patent system. The whole of the carcass was then placed in the cabinet, and the portal catheter was connected to the perfusion apparatus, care being taken to prevent air bubbles from entering the circulation.

The first 10–15 ml of venous blood was discarded and the height of the oxygenator was adjusted to give a hydrostatic pressure of about 40 cm, which produced a flow rate of 35–40 ml/min without causing the liver to swell. A perforated plastic hemisphere was placed over the liver and covered with saline-soaked swabs to prevent the surfaces of the lobes from becoming dry.

The whole operation took about 15 min. There was an interval of about 5 min between the portal-vein catheterization and the start of the perfusion. The heartbeat usually ceased during this interval, but an auricular fibrillation often persisted up to the time of insertion of the venous catheter. Slightly extended periods of hepatic

ischaemia produced no noticeable effects on the performance of the liver.

RESULTS AND DISCUSSION

Viability of the perfused liver preparation. Maintenance of the normal fresh reddish-brown hue of the liver was well correlated with the effectiveness of the preparation. A decline in the rate of flow of perfusate through the liver, for example, caused a rapid darkening of the tissue and a rise in the transaminase concentration in the perfusate. In preparations of good appearance, and normal perfusate flow, transaminase activities were unchanged (4.6–10.3 m-units/ml) for 90–120 min.

At the end of the perfusion sections of liver were taken at random, fixed in aqueous formaldehyde (4%, w/v), blocked in wax, sectioned and stained with haematoxylin and eosin. Tissue from freshly killed birds of the same age maintained under identical conditions was prepared in the same way to provide 'normal' tissue. Microscopic examination revealed only slight differences between the two tissues. The hepatic cells were well preserved in the perfused tissue, and the erythrocytes from the perfusate were evenly distributed throughout the sinusoids. Some dilatation of the hepatic capillaries was occasionally observed.

The change in the oxygen concentration of the perfusate across the liver was measured at intervals (20 min) throughout the perfusion by the method of Peters & Van Slyke (1932). No attempt was made to regulate the oxygen concentration, but experiments were rejected when the oxygen uptake of the tissue, calculated from the flow rate of the perfusate

and the weight of the liver, fell by more than 10% during the perfusion. The mean oxygen consumption during eight perfusions was 1.94 (range 1.48–2.34, S.E.M. 0.09) $\mu\text{mol/min per g}$ of tissue and venous blood still contained 2.0–3.5 vol. % of oxygen.

The criteria of microscopic and visual appearance, oxygen consumption and transaminase activity were satisfied in the present studies, but bile production, which is sometimes used as an index of viability (Hems *et al.* 1966) was not monitored because the extra time spent in cannulating the bile duct would have extended the period of hepatic ischaemia.

Lipogenesis from glucose and palmitate. Albumin-bound [9,10- $^3\text{H}_2$]palmitate (170 μCi) and [U- ^{14}C]-glucose (60 μCi) were added to the perfusates 30 min after the start of the perfusion, and at the end of the experiments the triglycerides and free fatty acids of the liver and perfusate were assayed for radioactivity. Livers from fed and starved chickens showed a considerable capacity to incorporate palmitate into triglycerides, but appreciable lipogenesis from glucose was observed only with livers from fed birds (Table 1). This substrate contributes to glycerol as well as fatty acids, and in one experiment the triglycerides were hydrolysed and the products assayed for specific radioactivity (Table 1).

The distribution of radioactivity in the lipids of the liver and the perfusate showed that considerable amounts of the triglyceride synthesized in the liver from both [^{14}C]glucose and [$^3\text{H}_2$]palmitate were released into the perfusate.

Glucose utilization by the perfused liver. Glucose uptake by the liver was calculated from changes in the concentration in the perfusate, and from the

Table 1. Incorporation of radioactivity from [U- ^{14}C]glucose and [9,10- $^3\text{H}_2$]palmitate into triglycerides and free fatty acids by perfused livers from fed and starved chickens.

Radioactive substrate (15 ml) was added as a single injection to the perfusate (300 ml) 30 min after the start of the perfusion.

Expt. no.	Radioactivity incorporated (% of that added)								Recovery of added radioactivity in lipids (%)	
	Perfusate				Liver					
	Triglycerides		Free fatty acids		Triglycerides		Free fatty acids			
	¹⁴ C	³ H	¹⁴ C	³ H	¹⁴ C	³ H	¹⁴ C	³ H		
Fed										
1	1.2	16.7	0.2	3.8	2.7	22.7	0.002	0.1	4.1	43.3
2	2.0 (1.1*)	18.2	0.1	19.1	6.1 (4.2*)	49.5	0.100	1.7	8.3	88.5
3	1.7	14.8	0.1	7.1	13.8	58.6	0.200	2.7	14.7	83.2
Starved										
1	0.1	12.5	0.1	48.2	0.2	28.5	0.009	1.2	0.4	90.4
2	0.2	6.0	0.1	15.9	0.2	25.3	0.007	2.3	0.5	49.5
3	0.1	8.5	0.2	24.1	0.3	23.7	0.009	1.9	0.6	58.2

* Values in parentheses show radioactivity in triglyceride fatty acids.

Table 2. *Uptake of glucose by the perfused livers from fed and starved chickens at various perfusate concentrations of glucose*

Radioactive substrate (15 ml) was added as a single injection to the perfusate (300 ml) 30 min after the start of the perfusion.

Expt. no.	Mean concn. of perfusate glucose (mg/100 ml)	Liver wt. (g)	Glucose uptake (mg/h per g of tissue)
Starved			
1	73	36.5	11.1
2	131	30.1	5.0
3	163	28.4	4.1
4	182	22.2	15.3
Fed			
1	222	33.5	3.8
2	247	36.9	8.1
3	402	47.9	8.1
4	556	57.2	9.7
5	696	55.7	12.2

Table 3. *Conversion of L(+)-[U-¹⁴C]lactate into glucose by the perfused liver from a fed chicken*

The L(+)-[U-¹⁴C]lactate (50 μ Ci) was added 30 min after the start of perfusion.

Time after start of perfusion (min)	Concn. of glucose (mg/100 ml)	Concn. of lactate (mg/100 ml)	Sp. radioactivity of glucose (μ Ci/g of C)	Sp. radioactivity of lactate (μ Ci/g of C)
0	104	36		
15	258	18		
29	364	19		
33	355	16	13.4	592
45	408	11		
60	436	8	15.1	182
75	517	9		
90	526	5	14.0	159
105	563	6		
120	586	5	16.8	149

decline in the specific radioactivity of glucose that occurred during the period over which measurements were made (30–150 min from start of infusion). The results from five experiments with livers from fed birds and four experiments with livers from starved birds are shown in Table 2. With livers from fed birds the rate of glucose uptake was dependent on the concentration in the perfusate, $[y(\text{glucose uptake}) = 2.68 + 0.0134x \text{ (glucose concentration)}; P < 0.05]$ but no correlation between these parameters was shown by the livers from starved birds. The much lower glucose concentrations obtained with the latter preparations, which reflected the lower glycogen contents in starved birds, prevented an adequate examination of the dependence of glucose uptake on concentration.

The values for glucose uptake by the liver, expressed in relation to body weight (mean value 5.5 mg/min per kg^{0.75}) were about 50% of those for total glucose uptake (11.0 mg/min per kg^{0.75})

measured earlier in older birds (Annison, Shrimpton & West, 1969).

Gluconeogenesis from lactate. Lactate concentrations in the perfusate were relatively high during the early stages of the perfusion, but declined slowly to 5–10 mg/100 ml during the first 60 min. The interrelations of lactate and glucose were examined using L(+)-[U-¹⁴C]lactate (50 μ Ci), which was added to the perfusate 30 min after the start of the perfusion. The results of an experiment with the liver from a fed bird are shown in Table 3. Comparison of the specific radioactivities of glucose and lactate showed substantial glucose synthesis from lactate. After 120 min less than 10% of the added radioactivity was present as lactate, and about 20% of the added radioactivity was accounted for as glucose. The relative constancy of the specific radioactivity of glucose over the period when the pool size was steadily increasing (Table 3) is evidence of the continuous contribution of lactate

Table 4. Incorporation of [1-¹⁴C]acetate into triglycerides and free fatty acids by perfused livers from fed and starved chickens

Expt. no.	Radioactivity incorporated (% of that added)				
	Perfusate		Liver		Total
	Triglycerides	Free fatty acids	Triglycerides	Free fatty acids	
Fed					
1	8.3	0.1	14.6	0.3	23.3
2	5.1	0.1	9.3	0.2	14.7
Starved					
1	1.1	<0.1	1.6	0.1	2.8
2	0.8	<0.1	1.5	0.1	2.4

to gluconeogenesis. The simultaneous decline in lactate concentration and specific radioactivity indicated the continuous utilization and production of lactate.

Liver glycogen. Rapid glycogenolysis occurred during every perfusion, irrespective of the efficiency of the preparation as assessed by flow rate, oxygen consumption, gluconeogenesis from lactate or transaminase activities in the perfusate. At the end of the perfusion glycogen contents rarely exceeded 0.2% (w/w).

Utilization and production of acetate. [1-¹⁴C]-Acetate (50 μ Ci) was added to the perfusate 30 min after the start of the perfusion, and the triglycerides and free fatty acids of the liver and perfusate were assayed for radioactivity after 120 min. The livers from fed chickens showed a greater capacity for lipid synthesis from acetate than did the starved livers (Table 4). Acetate concentrations in the perfusate remained relatively constant at 0.3–0.5 mmol/l in all experiments, suggesting continuous production by the liver. This was confirmed by the steady decline in the specific radioactivity of acetate, which allowed the rate of acetate production to be roughly calculated. Values of 62 and 63 μ g/h per g of liver were obtained for the two preparations from fed birds, and 7 and 12 μ g/h per g of liver for the two preparations from starved birds.

The production of endogenous acetate during the liver perfusions was clearly shown, but the results suggested that the liver is not a major site of production in the intact bird. Entry rates of acetate in fed birds of the size of the donor birds measured in earlier studies were 5–10 mg/min (Annison *et al.* 1969), which must be compared with acetate

production rates by the perfused liver of about 0.03 mg/min.

There was some transfer of radioactivity into glucose, and at the end of the perfusions the specific radioactivity of glucose was 5–10% of that of acetate in the four experiments.

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