Urate Synthesis in the Perfused Chick Liver

By EILEEN BARRATT, PETER J. BUTTERY* and K. NEIL BOORMAN

*Department of Applied Biochemistry and Nutrition, University of Nottingham School of Agriculture,
Sutton Bonington, Loughborough, Leics. LE12 5RD, U.K.*

(Received 22 March 1974)

Urate synthesis was studied in a perfused chicken liver preparation. The perfused liver had an ATP/ADP ratio of 0.29 ± 0.05 (6) compared with 0.34 ± 0.07 (10) in liver obtained from chicks under ether anaesthesia. Lactate/pyruvate ratios were 9.4 ± 1.7 (5) in the perfused liver and 14.8 ± 1.8 (5) in the rapidly sampled liver. Urate synthesis was only marginally stimulated by glycine, glutamine, aspartic acid or NH_4Cl , but significant increases were observed with phosphoribosyl pyrophosphate, aminoimidazolecarboxylic acid riboside, inosine, inosinic acid and xanthine. Urate synthesis from glycine, glutamine, NH_4Cl , asparagine, alanine, histidine and a mixture of 21 amino acids was obtained on inclusion of insulin in the perfusion medium. Evidence for the inclusion of the carbon of histidine into uric acid was obtained. Aspects of the energy consumption associated with the conversion of excess of amino acid into uric acid are considered.

Uric acid is the main nitrogenous excretory product in the bird, and in the chick the liver is believed to be the primary site of synthesis (Edson *et al.*, 1936). Isotopic work (Sonne *et al.*, 1946; Buchanan & Sonne, 1946; Sonne *et al.*, 1948; Buchanan *et al.*, 1948) has shown the precursors of uric acid to be glycine, glutamine, aspartate, formate and CO_2 .

Nutritional studies with the chick have emphasized the need for quantitative data relating urate output to the intake of its nitrogen precursors and in particular an estimate of the energy required for urate synthesis (see Solberg *et al.*, 1971). Some quantitative data have been obtained by using intact animals (Creek & Vasaitis, 1961; Featherston & Scholz, 1968; Bloomfield *et al.*, 1969; Karasawa *et al.*, 1973), but such techniques do not readily allow estimation of the energy required. The complexity of the biosynthetic pathway of purines also makes calculation of the energy utilized difficult. One aim of the present study was therefore to obtain a perfused liver preparation capable of producing uric acid from added substrate and to determine energy utilization by measuring O_2 consumption during synthesis.

Initial reports of this study have been published (Locke *et al.*, 1972; Buttery *et al.*, 1973).

Materials and Methods

Perfusion apparatus

The apparatus and the cabinet in which it was housed were based on those designed for rat liver perfusions by Hems *et al.* (1966). The temperatures of the cabinet and perfusion medium were maintained

at 40°C, the body temperature of birds. The total volume of perfusate was 200 ml. A mixture of $\text{O}_2 + \text{CO}_2$ (95:5) saturated with water vapour was continuously passed over the medium in the oxygenator.

Perfusion technique

Chick livers were perfused *in situ* with a medium of whole chicken blood (obtained, by using heparin as an anticoagulant, from cockerels of at least 10 weeks of age) diluted 1:2 with Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing heparin (5 i.u./ml). The whole blood (70 ml) was dialysed against Krebs–Ringer solution (5 litres) for 48 h at 4°C before use, to remove much of the free amino acids from the medium. After dilution of the whole blood, glucose was added to the perfusate to a final concentration of 1 mmol/100 ml. If necessary the medium was adjusted to pH 7.4 by gently bubbling CO_2 through it.

Chicks (male Rhode Island Red $\times$ Light Sussex, 3–6 weeks of age) were starved for 18 h before use. They were anaesthetized by a single injection of sodium pentobarbitone (30 mg/100 g body wt.) into the brachial vein. Livers were prepared for perfusion essentially by the method of Bickerstaffe *et al.* (1970). Modifications were adopted to simplify the operation. After exposure of the cardio-abdominal chamber, the inferior mesenteric vein was catheterized with a no. 16 Frankis–Evans needle. The tip of the catheter was pushed up into the portal vein. The needle was then ligatured and the trocar removed leaving the catheter in place. The supply of blood to the mesenteric vein was then occluded by ligaturing the vessel posterior to the cannula. Approx. 5 ml of perfusion

* To whom reprint requests should be addressed.

medium (at 40°C) was then immediately flushed through the cannula with a syringe. The pancreaticoduodenal and cocolygeo-mesenteric veins were tied off. The anterior vena cava and right and left cranial venae cavae were clamped off in turn with artery forceps. The pericardium was removed and a catheter of Portex (Hythe, Kent, U.K.) tubing, size pp 200, the tip of which had been heated and drawn out to a tube of approx. 2mm external diameter and cut off at an angle to form a sharp tip, was passed through the right atrium and its end aligned into a position where the two hepatic veins enter the atrium. The catheter was ligatured into position. The whole of the carcass, on a Perspex platform, was immediately placed in the perfusion cabinet and the portal catheter was connected to the apparatus. The first 10–15 ml of venous blood were discarded and then the outflow from the hepatic catheter was allowed to drip into the collecting vessel. The oxygenator was adjusted in height to give a hydrostatic pressure of 25–30 cm of water, which was sufficient to maintain a steady flow rate through the liver of 25–30 ml/min. The liver was kept moist throughout perfusion by placing a paper tissue soaked in 0.9% NaCl over the organ.

The complete operation took 5–7 min and an interval of no more than 3–4 min elapsed between catheterization of the portal vein and the start of perfusion.

Additions to the perfusate during a perfusion were made via the reservoir of the apparatus.

Sampling procedures

Samples (usually 1.0 ml) of perfusate were removed from the collecting vessel with a syringe, centrifuged at 3000 *g* for 10 min and the plasma was stored at –15°C until required.

Liver samples were taken by rapidly freezing *in situ* with clamps previously cooled in liquid N₂ (Wollenberger *et al.*, 1960). The frozen tissue was powdered under liquid N₂ in a pre-cooled mortar. The powder was denatured with ice-cold 0.6 M-HClO₄ and the supernatant solution neutralized to approx. pH 7.0 with 2 M-KOH. After being cooled in ice the KClO₄ precipitate was removed by centrifugation.

Chemicals

Uricase (urate–oxygen oxidoreductase, EC 1.7.3.3; 0.2–0.4 EC unit/ml), xanthine oxidase (xanthine–oxygen oxidoreductase, EC 1.2.3.2; 0.5–1.0 EC unit/ml), tetrahydrofolic acid (DL-5,6,7,8-tetrahydropteroyl-L-glutamic acid), 5-phosphoribosyl 1-pyrophosphate, 4-aminoimidazole-5-carboxamide riboside, IMP and inosine were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Pentobarbitone sodium (Nembutal; 60 mg/ml) was from Abbott Laboratories Ltd., Queenborough, Kent, U.K. Heparin (5000 i.u./ml) was from Boots Drug Co., Nottingham, U.K. Chemicals for determination of glucose, lactate,

pyruvate, ATP, ADP, glutamate–oxaloacetate transaminase (EC 2.6.1.1) and glutamate–pyruvate transaminase (EC 2.6.1.2) were purchased from Boehringer (London) Corp. Ltd., London W5 2TZ, U.K. Protosol, a tissue solubilizer, was obtained from New England Nuclear Corp. (Pilot Chemicals Division), Boston, Mass., U.S.A. Radioactive chemicals, [1-¹⁴C]glycine (240 mCi/mmol), amino[1-¹⁴C]isobutyrate (58 mCi/mmol), L-[U-¹⁴C]histidine (>270 mCi/mmol) and [³H]inulin (300 mCi/mmol), were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Scintillation-counting chemicals, PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene], were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. All amino acids were obtained from British Drug Houses, Poole, Dorset, U.K., and were of chromatographically homogeneous grade whenever possible. Insulin (24 i.u./mg; Sigma Chemical Co.) was of bovine origin and was dissolved in 154 mM-NaCl (4 mg/ml) by using a small quantity of 0.01 M-HCl if necessary.

Analytical procedures

Perfusate urate and xanthine were determined by specific enzyme techniques (Kalckar, 1947). Perfusion medium was spun in a bench centrifuge, the plasma removed and used immediately for the enzyme assay. For urate, 0.1 ml of plasma, 1 ml of glycine buffer (0.7 M-glycine adjusted to pH 9.4 with NaOH) and 6 ml of water were mixed and two 3 ml portions taken. To one portion was added 0.02 ml of uricase and to the other an equal volume of water. The extinction of samples was measured at 292 nm after 30 min. For total xanthines, 0.2 ml of plasma and 7 ml of potassium phosphate buffer (0.05 M-KH₂PO₄ adjusted to pH 7.4 with KOH) were mixed and two 3 ml portions taken. To one portion was added 0.02 ml of xanthine oxidase and to the other an equivalent volume of water. The samples were read at 292 nm after being left at room temperature for 60 min.

Liver uric acid was extracted by the method of Bokori (1965). The filtrate was neutralized with 1 M-NaOH to approximately pH 7.0 and 0.1 ml of the filtrate assayed for uric acid with uricase as for plasma samples.

Liver water content was measured by determining the loss in weight of the left lobe of the liver dried at 110°C for 48 h.

Perfusate pH value was measured by the insertion of a combined glass–calomel electrode at the appropriate point in the oxygenator.

Perfusate K⁺ concentrations were determined by flame photometry. Plasma samples were deproteinized with an equal volume of 1 M-HClO₄. The supernatant obtained after bench centrifugation was diluted 1:50 (v/v) with water.

Serum glutamate–oxaloacetate transaminase and

glutamate-pyruvate transaminase activities were determined colorimetrically with the aid of 2,4-dinitrophenylhydrazine by the method of Bergmeyer & Bernt (1965a).

Perfusate glucose concentrations were measured by a glucose oxidase-peroxidase method (Huggett & Nixon, 1957). It was necessary to precipitate the uric acid from the plasma before the glucose assay could be performed, since if large amounts of reducing agent (e.g. uric acid) are present in the sample they will compete with peroxidase for the H_2O_2 formed (Bergmeyer & Bernt, 1965b). Uric acid was precipitated from the medium as the mercuric salt by the method of Bergmann & Dikstein (1954), and the supernatant solution assayed for glucose. Perfusate and liver lactate concentrations were determined by using lactate dehydrogenase (Hohorst, 1965). Pyruvate concentrations in the perfused liver were measured by the method of Bücher *et al.* (1965).

ATP and ADP concentrations in the liver were measured on samples of rapidly fixed tissue by the methods of Adam (1965a,b).

O_2 consumption by the perfused liver was measured at intervals throughout the perfusion period by using a Natelson Microgasometer (Scientific Industries Inc., Springfield, Mass., U.S.A.) by the method of Van Slyke & Neill (1924). Inflowing oxygenated perfusate samples were taken with a syringe from the reservoir at the bottom of the oxygenator and outflowing venous samples from the venous catheter close to where it leaves the right atrium. This avoided any loss of O_2 from the perfusate through the walls of the polyvinyl tubing. Samples were introduced into the microgasometer without delay, since storage of samples in the syringe was found to lead to changes in the gas content.

The appearance of the liver was examined at the end of the perfusion period both with the naked eye and under the electron microscope. For the latter, the liver was fixed in buffered glutaraldehyde followed by OsO_4 . Sections were cut and fixed in uranyl acetate and lead citrate (Reynolds, 1963).

Radioactive isotope counting techniques

Counting of ^{14}C and 3H was carried out in a Tracerlab liquid-scintillation spectrometer, Corumatic 200. A mixture of 5.0g of PPO and 0.5g of POPOP dissolved in 1 litre of toluene was used as the scintillant. The final volume of mixtures of sample and scintillator was 15ml. Quench correction was carried out by an external standard. [^{14}C]Urate was determined in perfusate samples by precipitation of uric acid from the medium by the method of Bergmann & Dikstein (1954). The precipitate from 1ml of plasma was washed several times in a solution of the non-radioactive precursor (0.5mm) of uric acid used in the particular experiment. The precipitate was dissolved in 1.5ml of ethanolamine. To 0.5ml of this solution 1.5ml

of methanol and 13ml of scintillator was added and the mixture assayed for radioactivity.

Amino[^{14}C]isobutyrate uptake by the perfused liver

This was determined by a dual-labelling system. The ratio amino[^{14}C]isobutyrate/[3H]inulin in the perfusate plasma was compared with that in the perfused liver to give an absolute measure of ^{14}C uptake, since inulin is not taken up to any significant extent by the liver. The perfusate was assayed for radioactivity by digestion of 0.1ml of plasma in 1ml of Protosol tissue solubilizer in hermetically sealed vessels at 55°C for 24h in the dark. The solution was diluted 1:4 (v/v) with a 1:1 (v/v) mixture of toluene and methanol. To 0.5ml of this solution 14.5ml of scintillation solution was added and the mixture stored in the dark for 48h at room temperature before being counted for radioactivity to minimize the effects of chemiluminescence. Liver samples (about 100mg wet wt.) were frozen and then digested in 1ml of Protosol in a similar manner to that used for perfusate samples. The resulting solution was diluted 1:10 (v/v) with a 1:1 (v/v) mixture of toluene and methanol. To 1.0ml of this solution, 14ml of scintillation solution was added.

Presentation of data

All results are presented as means $\pm$ S.E.M. with numbers of observations in parentheses, whenever possible.

Results

Liver viability

Maintenance of the normal appearance of the liver was well correlated with its ability to synthesize uric acid. There were no visible signs of oedema. Electron-microscopic examination of the perfused liver tissue after 150min perfusion revealed little change in ultrastructure apart from a slight dilation of the rough endoplasmic reticulum. Visual inspection of the electron micrographs indicated that the shape of the mitochondria and the glycogen content of the cells were unchanged. No evidence of autophagic vacuoles was observed (Abraham *et al.*, 1968). The water content of the perfused liver [75.3 ± 0.2 (6)%] after 150min perfusion increased slightly compared with the water content of the liver *in vivo* [74.1 ± 0.3 (4)%]. Initial concentrations of perfusate K^+ of 6.9 ± 0.24 (6) mequiv./litre fell during the first 20–30min of perfusions, after which the concentration remained at 6.11 ± 0.35 (6) mequiv./litre for periods of up to 150min. Perfusate pH (7.4–7.5) was unchanged during perfusion.

Transaminase activities in the perfusate changed little in preparations of good appearance. Glutamate-oxaloacetate aminotransferase increased during the 150min perfusions from 19.5 ± 3.4 (10) to

Table 1. *Lactate/pyruvate and ATP/ADP ratios in vivo and in the perfused liver*

Livers of chicks (starved for 18h) were perfused with standard perfusion medium as described in the text for 150min. Samples of liver were taken by a rapid-freezing technique. Unperfused control values were obtained from livers of chicks and rats *in vivo* (starved for 18h) and removed under ether anaesthesia. Values are means $\pm$ S.E.M. with numbers of samples in parentheses.

	Content (μ mol/100 g of liver)	
	<i>In vivo</i>	Perfused (150min)
Lactate (chick)	118 $\pm$ 15 (5)	85 $\pm$ 3 (5)
Pyruvate (chick)	7.8 $\pm$ 1.0 (5)	8.9 $\pm$ 1.1 (5)
Lactate/pyruvate	14.8 $\pm$ 1.8 (5)	9.4 $\pm$ 1.7 (5)
ATP (chick)	58 $\pm$ 7 (10)	49 $\pm$ 6 (6)
ADP (chick)	160 $\pm$ 12 (10)	140 $\pm$ 9 (6)
ATP/ADP (chick)	0.34 $\pm$ 0.07 (10)	0.29 $\pm$ 0.05 (6)
ATP (rat)	240 $\pm$ 10 (3)	
ADP (rat)	95 $\pm$ 8 (3)	
ATP/ADP (rat)	2.50 $\pm$ 0.10 (3)	
ATP (chick+rat)*	135 $\pm$ 9 (3)	
ADP (chick+rat)*	122 $\pm$ 8 (3)	

* Equal volumes of rat and chick liver extracts were taken; expected ATP value 149, ADP value 127.

34.3 $\pm$ 3.6 (10) munits/ml. The increase was non-linear, becoming more rapid towards the end of the perfusion period. In preparations of poor appearance the increase was much more rapid, increases of up to 40 munits of glutamate-oxaloacetate aminotransferase/h per ml being recorded. Glutamate-pyruvate aminotransferase activity in the perfusate was very low [0.5 $\pm$ 0.2 (10) munit/ml] and changed little during the course of 150 min perfusion [0.7 $\pm$ 0.4 (10) munit/ml at 150 min].

Lactate/pyruvate ratios in the perfused liver after 150 min perfusion [9.4 $\pm$ 1.7 (5)] were lower than values *in vivo* [14.8 $\pm$ 1.8 (5)] (Table 1). Perfusate lactate concentrations fell from 176 $\pm$ 9 (6) to 53 $\pm$ 9 (6) μ mol/100 ml during 150 min perfusion. The decrease was rapid during the first 30 min of perfusion and then slowed, until at 70 min no further changes were recorded.

Concentrations of perfusate glucose [1.28 $\pm$ 0.05 (7) mmol/100 ml] rose during the first 30 min of perfusion to 1.84 $\pm$ 0.07 (7) mmol/100 ml and then fell so that after 150 min concentrations of 1.25 $\pm$ 0.05 mmol/100 ml were recorded. Thus there was little overall change during the course of perfusion. The O₂ content of fully oxygenated medium was 99.5 $\pm$ 1.9 (14) ml/litre and in the venous perfusate was 45.1 $\pm$ 8.2 (10) ml/litre; thus the perfused liver took up about 54% of the O₂ from the medium. The mean O₂ consumption without added substrate was 1.70 $\pm$ 0.09 (8) μ mol/min per g of liver. The rate changed very little during the

course of a single perfusion, after the first few minutes of perfusion when a significantly ($P < 0.05$) increased rate of O₂ consumption was observed (2.21 $\pm$ 0.14 μ mol/min per g of liver).

ATP/ADP ratios found in the perfused chick liver after 150 min perfusion [0.29 $\pm$ 0.05 (6)] were similar to those found in the chick liver *in vivo* [0.34 $\pm$ 0.07 (10)], although the overall concentration of nucleotides was slightly decreased after perfusion. These ratios are much lower than those found in the rat liver *in vivo* [2.5 $\pm$ 0.10 (3)]. A mixture of equal volumes of rat and chick liver extracts, prepared in an identical manner, showed the expected values for ATP and ADP concentrations in the mixture, indicating further that the methods of assay used were satisfactory (Table 1).

Endogenous uric acid production in perfused liver

Livers of chicks were perfused for 150 min. Perfusate samples (1.0 ml) were taken at regular time-intervals (every 10 or 15 min) and the plasma was assayed for uric acid and the endogenous rate of uric

Fig. 1. *Effect of insulin on uric acid production in the presence (▲) and absence (■) of added glycine*

Livers from starved chicks (18 h) were perfused with 200 ml of medium as described in the text. Nine experiments were conducted. Glycine (1 mmol) was added to the perfusate after 50 min in six perfusions, arrow (1). Insulin (0.1 i.u.) was added at 90 min, arrow (2) to all perfusions. Each point represents the mean $\pm$ S.E.M. of three, six or nine determinations as appropriate. The lines have been fitted to the data by eye.

acid output determined. A rapid initial output of uric acid was observed for approximately the first 15 min of perfusion. This rate was very variable [8.21–16.11 (20) $\mu\text{mol/h}$ per g of liver]. After this period the rate declined to a mean linear rate of 4.69 ± 0.47 (20) $\mu\text{mol/h}$ per g of liver for periods up to 150 min (Fig. 1). This rate also showed considerable variation between individual livers. After 150 min a decline in this rate was sometimes observed and marked a general decline in metabolic activity as judged by the tests of perfusion viability used in this study. At the end of the perfusion period, perfusate uric acid concentrations were between 120 and 210 $\mu\text{mol}/100\text{ml}$; concentrations varied with liver weight and the treatment used during the perfusion. In the pigeon liver, lack of xanthine dehydrogenase causes the output of hypoxanthine from the liver and this is oxidized to uric acid in the kidney (Edson *et al.*, 1936). Hypoxanthine and xanthine were therefore measured in the chick liver perfusate, but no significant change from the initial concentration of total xanthine [5.92 ± 0.13 (5) $\mu\text{mol}/100\text{ml}$] was observed.

Uric acid production from uric acid precursors

A series of experiments was carried out in which the liver was perfused for 50 min as described above and then glycine, glutamine, aspartate and NH_4Cl (1 mmol) were added individually to the perfusate. The plasma was assayed for uric acid and xanthines. These amino acids were chosen since they are known to be directly involved in the biosynthetic pathway of uric acid (Hartman, 1970). However, no change was observed from the initial rate of uric acid output [4.74 ± 0.42 (12) $\mu\text{mol/h}$ per g of liver] when these substrates were added. Perfusate xanthine concentrations were also unchanged [5.41 ± 0.21 (12) $\mu\text{mol/h}$ per g of liver]. There was also no evidence that the

addition of these substrates had any effect on the concentration of urate in the liver itself. Uric acid concentrations in the perfused liver after 150 min perfusion without added substrate [170.2 ± 8.6 (6) $\mu\text{mol}/100\text{g}$ of liver] were significantly ($P < 0.05$) higher than the concentrations *in vivo* [144.6 ± 6.2 (3)]. However, these concentrations in the perfused liver were not significantly affected by the addition of glycine (5 mm) plus glutamine (5 mm) to the medium, when concentrations of liver uric acid of 177.3 ± 7.3 (5) $\mu\text{mol}/100\text{g}$ of liver were recorded. The addition of serine (5 mm) plus tetrahydrofolic acid (0.26 mm) as a source of C_1 units (Yoshida & Kikuchi, 1971) also had no effect on uric acid production.

When 2 μCi of [$1\text{-}^{14}\text{C}$]glycine (2 $\mu\text{Ci}/\text{mmol}$) was added to the perfusate after 50 min perfusion small amounts of radioactivity were found in the uric acid produced. The specific radioactivity of [^{14}C]uric acid was 262 ± 23 (4) d.p.m./ μmol , compared with the specific radioactivity of [^{14}C]glycine added, 4440 d.p.m./ μmol .

The effect of various uric acid intermediates on uric acid output by the perfused liver, when added to the perfusate at 50 min, is shown in Table 2. Those intermediates occurring earlier in the biosynthetic pathway, such as phosphoribosyl pyrophosphate, caused less stimulation than those occurring later in the biosynthetic pathway, such as xanthine. The intermediates were added in 50 mg quantities, which gave various molar amounts. However, the quantity added did not appear to affect significantly the rate of stimulation as shown with aminoimidazolecarboxamide riboside in Table 2, although when the addition of 20 mg of aminoimidazolecarboxamide riboside (0.30 mm) to the perfusate was made the increased rate of uric acid synthesis was only observed for approx. 60 min and not until the end of the perfusion

Table 2. Effect of uric acid intermediates on the output of uric acid by the perfused chick liver

Livers of chicks (starved for 18 h) were perfused with standard perfusion medium (200 ml) for 150 min as described in the text. Uric acid intermediates were added to the perfusate in 50 mg quantities after 50 min perfusion. Samples of perfusate taken at regular time-intervals were assayed for uric acid. The extra urate synthesized was calculated in each experiment from the measurement of the uric acid production before and after the addition of substrates. Values are means $\pm$ s.e.m. Initial rate of urate output refers to the rate of uric acid output observed after the rapid rate of uric acid synthesis (approximately from 0 to 15 min) has ceased (see Fig. 1).

Uric acid intermediate (final concn.)	No. of perfusions	Initial rate of urate output ($\mu\text{mol/h}$ per g of liver)	Rate of urate output after intermediate added ($\mu\text{mol/h}$ per g of liver)	Increase in uric acid output (%)
Phosphoribosyl pyrophosphate (0.55 mm)	4	3.21 ± 0.47	5.41 ± 0.77	72 ± 10
Aminoimidazolecarboxylic acid riboside (0.75 mm)	4	3.21 ± 0.52	10.00 ± 0.82	210 ± 30
(0.30 mm)	3	3.71 ± 0.47	11.03 ± 1.04	200 ± 19
(1.05 mm)	3	4.05 ± 0.44	12.10 ± 1.31	205 ± 22
IMP	5	4.82 ± 0.47	18.45 ± 2.35	303 ± 6
Inosine	3	4.46 ± 0.49	18.39 ± 1.84	314 ± 12
Xanthine	4	3.57 ± 0.24	15.77 ± 1.79	298 ± 34

Table 3. *Effect of L-amino acids on the output of urate by the perfused chick liver, in the presence of insulin*

Livers of chicks (starved for 18 h) were perfused with standard perfusion medium (200 ml) for 150 min as described in the text. Amino acids (5 mM) were added to the perfusate after 50 min of perfusion. Insulin (0.1 i.u.) was added to the perfusate at 30 min intervals. Perfusate samples taken at regular time-intervals were assayed for uric acid. Each value is mean $\pm$ s.e.m. 'Initial rate of urate output' is explained in Table 2. For the 'plasma amino acid mixture', L-amino acids were added such that the initial perfusate concentration was as follows for each amino acid (μ M): arginine, 220; asparagine, 38; aspartate, 38; threonine, 290; serine, 290; glutamate, 190; glycine, 430; alanine, 480; valine, 200; methionine, 70; isoleucine, 90; leucine, 190; tyrosine, 38; phenylalanine, 80; histidine, 90; lysine, 480; proline, 242; cysteine, 38; glutamine, 290; tryptophan, 69. Thus the concentrations were similar to those found in plasma.

L-Amino acid added (5 mM)	No. of observations	Initial rate of urate output (μ mol/h per g of liver)	Rate of urate output after addition of amino acid (μ mol/h per g of liver)	Increase in urate output (%)
Glycine	4	5.20 $\pm$ 0.82	12.14 $\pm$ 1.41	125 $\pm$ 5
Glutamine	4	5.18 $\pm$ 0.77	7.91 $\pm$ 1.23	62 $\pm$ 4
Glycine + glutamine	5	4.16 $\pm$ 0.29	10.00 $\pm$ 0.47	140 $\pm$ 4
NH ₄ Cl	3	4.64 $\pm$ 0.24	5.71 $\pm$ 0.47	21 $\pm$ 2
Asparagine	3	3.21 $\pm$ 0.65	5.06 $\pm$ 1.04	62 $\pm$ 1
Alanine	7	4.34 $\pm$ 0.41	9.28 $\pm$ 0.49	122 $\pm$ 7
Histidine	5	3.15 $\pm$ 0.59	6.33 $\pm$ 0.94	101 $\pm$ 4
Aspartate	3	3.88 $\pm$ 0.48	*	
Arginine	3	4.72 $\pm$ 0.64	*	
Cysteine	3	4.81 $\pm$ 0.51	*	
Glutamate	3	5.01 $\pm$ 0.54	*	
Isoleucine	3	3.97 $\pm$ 0.48	4.11 $\pm$ 0.69	3 $\pm$ 2
Leucine	3	3.68 $\pm$ 0.47	*	
Lysine	3	4.61 $\pm$ 0.21	*	
Methionine	3	4.21 $\pm$ 0.38	4.40 $\pm$ 0.54	5 $\pm$ 3
Phenylalanine	3	4.23 $\pm$ 0.51	*	
Proline	3	4.84 $\pm$ 0.29	*	
Serine	3	4.51 $\pm$ 0.34	*	
Threonine	3	4.34 $\pm$ 0.63	4.50 $\pm$ 0.79	4 $\pm$ 3
Tryptophan	3	4.91 $\pm$ 1.40	5.00 $\pm$ 0.77	3 $\pm$ 1
Tyrosine	3	3.98 $\pm$ 0.81	*	
Valine	3	4.09 $\pm$ 0.54	*	
Plasma amino acid mixture	3	3.80 $\pm$ 0.24	10.90 $\pm$ 0.65	190 $\pm$ 4

* No detectable change in output on addition of the amino acid.

period (150 min), as with the higher concentrations of aminoimidazolecarboxamide riboside.

Urate output by the perfused liver in the presence of insulin

The failure of the perfused chick liver to produce uric acid from its amino acid precursors led to the search for a possible explanation. This produced the idea that the hormonal status of the organ may be inadequate. Insulin appears to be particularly important in nitrogen metabolism in perfused organs (Mondon & Mortimore, 1967; Miller & Griffin, 1972).

The addition of insulin to the perfusate (0.1 i.u. at 30 min intervals) had no effect on the endogenous rate of uric acid production. The effect of insulin on urate synthesis from glycine is shown in Fig. 1. The effect of amino acids on urate production in the presence of insulin is shown in Table 3. These experiments were carried out under conditions identical with those in the absence of insulin, as discussed above. Glycine

appeared to be the most effective promoter of uric acid synthesis in the perfusion system. High rates of stimulation were also observed with alanine and histidine. Glutamine and asparagine produced identical rates of stimulation. Aspartate, a known precursor of uric acid, had no effect on uric acid synthesis.

To determine in what manner histidine stimulated uric acid production studies using ¹⁴C were undertaken. When 2 μ Ci of L-[U-¹⁴C]histidine (2 μ Ci/mmol) was added to the perfusate after 50 min perfusion the specific radioactivity of the [¹⁴C]uric acid produced by the liver was 3439 $\pm$ 210 (4) d.p.m./ μ mol, compared with a specific radioactivity of 5079 $\pm$ 210 (4) d.p.m./ μ mol of the added [¹⁴C]histidine. Endogenous rates of uric acid production were subtracted before calculation of the specific radioactivity of the uric acid produced on the addition of histidine. The ratio [¹⁴C]uric acid/[¹⁴C]histidine was 0.677 $\pm$ 0.022 (4), indicating that histidine provided about two-thirds of the carbon skeleton of the uric acid molecule.

The presence of insulin in the medium had no significant effect on the rates of stimulation of uric acid synthesis from uric acid intermediates compared with those in Table 2.

Effects of insulin in the perfused chick liver system

The addition of insulin to the medium was not directly responsible for the increase in uric acid production from amino acids, but appeared to have an effect on the utilization of the amino acid for uric acid synthesis by the perfused liver (Fig. 1). The addition of insulin in the absence of glycine had no effect on uric acid output.

Insulin is reported to affect the concentration of various metabolites, e.g. inorganic phosphorus (Kestens *et al.*, 1963), K^+ (Burton *et al.*, 1967) and glucose and lactate (Mortimore *et al.*, 1967) in the perfusate of rat liver perfusion systems. In these experiments ATP/ADP ratios of the liver in the presence of insulin [0.31 ± 0.05 (4)] were not significantly different from those when insulin was absent. Similarly, as in the absence of insulin, perfusate K^+ concentrations fell from 6.84 ± 0.19 (3) to 5.91 ± 0.24 (3) mequiv./litre during 150 min perfusions; perfusate lactate concentrations fell from 185 ± 17 (3) to 60 ± 10 (3) $\mu\text{mol}/100\text{ml}$; and glucose concentrations followed a similar pattern to those in the absence of insulin, rising from an initial value of 1.32 ± 0.07 (3) to 1.81 ± 0.09 (3) $\text{mmol}/100\text{ml}$.

The uptake of 2-amino[1- ^{14}C]isobutyric acid by the liver was significantly ($P < 0.01$) greater after 90 min of perfusion in the presence of insulin than in its absence, as indicated by the $^{14}\text{C}/^3\text{H}$ ratios in the perfusate and in digested samples of liver tissue when 2 μCi of 2-amino[1- ^{14}C]isobutyric acid (2 $\mu\text{Ci}/\text{mmol}$) and 10 μCi of [^3H]inulin (0.125 $\mu\text{Ci}/\text{mg}$) had been added simultaneously, 30 min after the start of the

perfusion. An uptake of amino[^{14}C]isobutyric acid of 2045 ± 208 (4) d.p.m./min per g of liver was recorded in the presence of insulin compared with an uptake of 914 ± 113 (4) d.p.m./min per g in its absence.

Oxygen consumption during uric acid synthesis

To estimate the energy cost of uric acid synthesis O_2 -consumption measurements of the perfused liver were made. An increased uric acid production was induced in the perfused liver by the addition of 1 mmol of glycine or 1 mmol of glutamine to the perfusate after 60 min perfusion in the presence of insulin. The observed increase in O_2 consumption that occurred was then measured and compared with the initial rates of O_2 consumption. Samples of oxygenated blood were taken at 30 and 70 min and of venous blood at 50 and 90 min. The synthesis of uric acid from glycine or glutamine showed different requirements for O_2 (Table 4). The synthesis of 1 mol of uric acid from glycine required 1.49 ± 0.03 (5) mol of O_2 and from glutamine 1.16 ± 0.03 (5) mol of O_2 . This difference was highly significant ($P < 0.001$). Assuming that the basal ATP production and utilization was not altered on addition of glycine or glutamine and by assuming a P/O ratio of 3, it is possible to estimate the extra energy required for the extra uric acid synthesis in terms of the amount of ATP required (see Table 4).

Discussion

Liver viability

The results indicated that the liver functioned satisfactorily for periods up to 150 min. The appearance seemed to be a particularly useful criterion of perfusion viability.

Measurements of perfusate K^+ concentration,

Table 4. *Oxygen consumption during increased urate synthesis, stimulated by the addition of amino acids to the perfusate containing insulin*

Livers of chicks (starved for 18 h) were perfused for 150 min with standard medium to which insulin (0.1 i.u.) was added as described in the text. Glycine (1 mmol) and glutamine (1 mmol) were added to the perfusate after 60 min perfusion. The O_2 content of the 'arterial' perfusate was measured at 30 and 70 min and of venous blood at 50 and 90 min. Flow rates of the perfusate (25–30 ml/min) were measured simultaneously with of venous sampling. Perfusate was assayed for urate at regular time-intervals. The extra O_2 used was calculated for each perfusion from the measurement of the O_2 uptake before and after the addition of substrates. Results are expressed as means $\pm$ S.E.M.

Amino acid added (1 mmol)	No. of perfusions	Initial rate of urate synthesis ($\mu\text{mol}/\text{h}$ per g of liver)	Increase in urate production due to amino acid addition ($\mu\text{mol}/\text{h}$ per g of liver)	Initial rate of O_2 consumption ($\mu\text{mol}/\text{h}$ per g of liver)	Increase in O_2 consumption due to amino acid addition ($\mu\text{mol}/\text{h}$ per g of liver)	O_2 required to synthesize 1 mol of urate (mol)	ATP equivalent assuming P/O = 3 (mol)
Glycine	5	4.64 ± 0.38	$5.70 \pm 0.40^\dagger$	108.0 ± 3.0	$8.22 \pm 0.54^\dagger$	1.49 ± 0.03	8.97 ± 0.16
Glutamine	5	4.79 ± 0.35	$2.68 \pm 0.33^*$	104.8 ± 2.2	$3.71 \pm 0.41^*$	1.16 ± 0.03	6.99 ± 0.16

† Increase significantly different from zero, $P < 0.001$.

* Increase significantly different from zero, $P < 0.05$.

activities of transaminases and pH value indicated little change in cell permeability. The leakage of glutamate-oxaloacetate aminotransferase into the perfusate was small compared with that observed in unsatisfactory perfusions, as judged by the appearance of the liver. Serum glutamate-pyruvate aminotransferase activities would appear to be an unsatisfactory test for estimating liver damage in the chick owing to the low activity of this enzyme in this species.

The low lactate/pyruvate ratios in the perfused liver indicated that the intracellular redox state was not reduced. Lactate accumulation during the anoxic period of the operation probably accounted for the high initial concentrations observed in the perfusate.

O₂ uptake was extremely constant during the course of the perfusion, after the first few minutes where the high values recorded were probably due to an increased O₂ utilization necessary to metabolize intracellular lactate, which had accumulated during the operation. The rates of O₂ uptake observed (1.34–2.29 $\mu\text{mol/min per g}$ of liver) were similar to those observed in the perfused chick liver by Bickerstaffe *et al.* (1970) (1.48–2.34 $\mu\text{mol/min per g}$ of liver).

ATP/ADP ratios were very low both *in vivo* and in the perfused chick liver compared with the ratio in the rat liver *in vivo*. It might be possible to explain the low ATP/ADP ratio in the chick liver *in vivo* by the fact that, since the chick has no diaphragm opening of the abdomen, to freeze-clamp a portion of liver may result immediately in a hypoxic state of the tissue. However, this would not explain the low ATP/ADP ratios in the perfused chick liver, since the tissue was freeze-clamped while being provided with an external source of O₂. Söhling *et al.* (1973) have reported a low ATP/ADP ratio in the isolated perfused pigeon liver (0.82). They could offer no explanation for the low ratio and had no evidence that it resulted from hypoxia of the organ. They also found that the pigeon liver could perform very high rates of gluconeogenesis in the presence of very low tissue ATP/ADP ratios.

When the perfusion period was extended beyond 150 min adverse changes such as a rise in perfusate K⁺ concentrations and glutamate-oxaloacetate aminotransferase activity and a decline in the rate of O₂ consumption often occurred. These changes were on many occasions accompanied by a fall in the flow rate.

Uric acid output in the absence of insulin

The endogenous rate of uric acid output (0.078 $\mu\text{mol/min per g}$ of liver) by the perfused chick liver was similar to endogenous rates of urea production in the perfused rat liver recorded by Hems *et al.* (1966) (0.10 $\mu\text{mol/min per g}$ of liver) and Bloxam (1971) (0.13 $\mu\text{mol/min per g}$ of liver). Endogenous rates of uric acid output in the chick *in vivo* (anaesthetized) are 0.19 ± 0.02 (20) $\mu\text{mol/min per g}$ of liver (E. Barratt, P. J. Buttery & K. N. Boorman, unpublished work). The high rate of uric acid output

during the first 15 min of perfusion is probably accounted for by a washing out of liver uric acid by the perfusate and synthesis from metabolites that had accumulated during the operation.

The inability of the perfused chick liver to produce uric acid from its precursor amino acids was surprising, since these amino acids have been shown to stimulate uric acid synthesis in chick liver slices and the chick *in vivo* (E. Barratt, P. J. Buttery & K. N. Boorman, unpublished work). Studies with [¹⁴C]-glycine indicated that exogenous supplies of this amino acid acted as a precursor for only approx. 6% of the uric acid formed. There was no evidence of any xanthine output by the chick liver in these circumstances, indicating adequate activity of xanthine dehydrogenase in the chick liver to oxidize any hypoxanthine formed to uric acid. In the same system, uric acid synthesis from more complex intermediates was observed. Xanthine, inosine and IMP increased uric acid synthesis approx. threefold, and all these increases were significantly higher ($P < 0.05$; $P < 0.01$) than those observed with aminoimidazole carboxamide riboside and phosphoribosyl pyrophosphate respectively. Xanthine, inosine and IMP do not require any further substrate for uric acid synthesis, whereas aminoimidazolecarboxamide riboside requires a C₁ source and phosphoribosyl pyrophosphate requires two C₁ sources in addition to glycine and glutamine. It may be suggested that the differences in the rate of stimulation observed between these intermediates may be due to differences in uptake of these large molecules, although the non-availability of substrates within the liver cannot be excluded.

Urate output in the presence of insulin

Under conditions which stimulate amino acid uptake (i.e. the presence of insulin), glycine, glutamine, asparagine, NH₄Cl, alanine and histidine all stimulated uric acid output by the perfused chick liver. The differences in the extent of the stimulation given by these substances would be expected to reflect not only their role in the purine biosynthetic pathway but also the extent to which they were taken up by the liver. Mammalian cells tend to concentrate amino acids with polar side chains or no side chains more readily than amino acids with bulky, branched, apolar chains (Christensen, 1968). Glycine appeared to be the most effective promoter for uric acid synthesis in the chick liver; it is an essential amino acid to the chick and is incorporated as a unit into the uric acid molecule (Buchanan & Sonne, 1946), and there is also evidence that it may act as a source of the C₁ units necessary for uric acid synthesis (Yoshida & Kikuchi, 1971). Glutamine caused less stimulation of uric acid synthesis than did glycine; however, it is probable that glutamine can be readily synthesized, since the activity of glutamine synthetase (EC 6.3.1.2) is high in the chick liver (Katunuma *et al.*, 1970). Only a small

stimulation of uric acid synthesis was observed on the addition of NH_4Cl to the perfusate. Ammonia can replace glutamine as an amide donor in the purine ring (Hartman, 1963; Mizobuchi *et al.*, 1968). In the chick liver ammonia has also been shown to participate as an alternative to glutamine during the reaction for the synthesis of phosphoribosylamine, the initial step in purine synthesis (Reem, 1968). It appears that these reactions do not occur readily in the perfused chick liver. Asparagine produced a similar stimulation of uric acid synthesis to that observed with glutamine. The formation of glutamine from asparagine has been reported to take place via hydrolysis of asparagine to yield ammonia (T'ing Sen, 1959), although the presence of a transamination reaction has been suggested (Lestrovaya, 1954). However, if the former were the case in the perfused chick liver then it might be expected that the stimulation of uric acid observed with asparagine would be similar to that observed with NH_4Cl , since ammonia is readily taken up by the liver, at least in the rat (Lund *et al.*, 1970). However, the equivalence of NH_4Cl and asparagine was not observed, an observation that would tend to support the view that a transamination reaction between asparagine and glutamine may occur in the chick liver. The fact that glutamine and asparagine stimulated uric acid synthesis, whereas glutamate and aspartate did not, can perhaps be explained by the lack of uptake of these latter two amino acids. The uptake of dicarboxylic acids by the cell is known to be relatively slow (Wu, 1963).

Alanine produced a similar rate of uric acid output to that observed with glycine, though there is no evidence for the direct incorporation of this amino acid into uric acid. It may provide an energy source via pyruvate as well as a nitrogen source.

The studies using ^{14}C indicated that histidine provides part of the carbon skeleton of uric acid. It may also provide nitrogen atoms, but this was not investigated. The molecular structures of L-histidine and uric acid are very similar. Both molecules require phosphoribosyl pyrophosphate for synthesis and both include an imidazole ring, and the possibility of histidine giving rise to uric acid directly has been reported by Ackroyd & Hopkins (1916) and Rose & Cook (1925). However, when Barnes & Schoenheimer (1943) gave ^{15}N histidine to rats no appreciable amounts of the isotope were found in allantoin, uric acid or other purines.

Role of insulin in uric acid synthesis in the perfused chick liver

The increase in uric acid synthesis in the presence of insulin was unique to the addition of amino acids to the perfusate and was not observed on the addition of other uric acid intermediates. Insulin appeared to enhance the uptake of amino acids by the liver, as

indicated by studies using amino- ^{14}C isobutyrate. Miller & Griffin (1972) have indicated that the uptake of aminoisobutyrate by the perfused liver is paralleled by a less marked uptake of natural amino acids. Mondon & Mortimore (1967) showed a similar increased uptake of amino acids in the presence of insulin by the perfused rat liver. They suggested that insulin facilitated the use of individual amino acids for urea synthesis as well as facilitating their uptake. Thus a similar situation may be expected in the perfused chick liver with respect to uric acid synthesis. Since NH_4Cl did not stimulate uric acid output in the absence of insulin it may also be expected that insulin enhances the uptake of this substrate. This would appear to be possible, since insulin is known to enhance the uptake of K^+ by the liver (Kestens *et al.*, 1963) and that there are similarities between the transport of K^+ and ammonia (Lund *et al.*, 1970). The effect of insulin on amino acid transport appears to differ with individual amino acids (Oxender & Christensen, 1963); this may account for some of the variation in the response to different amino acids shown in Table 3. Insulin did not appear to have any effect on other metabolites measured, and did not appear to exert its effect by maintaining a sufficient concentration of ATP. If this had been so it might have been expected that a stimulation of uric acid synthesis would have been observed with phosphoribosyl pyrophosphate plus insulin in the perfusion system, above that observed with phosphoribosyl pyrophosphate alone.

Oxygen consumption associated with uric acid production

When glycine or glutamine was added to the perfusate there was an increase in uric acid production and an increase in O_2 uptake equivalent to the production of 9 mol of ATP/mol of uric acid for glycine and 7 mol of ATP/mol of uric acid when glutamine was the substrate. This difference may be accounted for by the need for glutamine synthesis with glycine. The difference between the ATP requirement of uric acid synthesis from glutamine suggested by the accepted metabolic pathway for uric acid synthesis (Hartman, 1970), and the increased value obtained in the present study, may be due to the stimulation of other metabolic pathways such as the synthesis of the required C_1 units. Studies on the synthesis of urea by the perfused mammalian liver have yielded higher than expected O_2 consumptions (Lundsgaard, 1942; Hems *et al.*, 1966).

E. B. was in receipt of a Ministry of Agriculture, Fisheries and Food Studentship. Helpful discussions with Dr. D. B. Lindsay are gratefully acknowledged.

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