Rate-Limiting Factors in Urate Synthesis and Gluconeogenesis in Avian Liver

By JAMES P. MAPES* and HANS A. KREBS

Metabolic Research Laboratory, Nuffield Department of Clinical Medicine,
Radcliffe Infirmary, Oxford OX2 6HE, U.K.

(Received 15 August 1977)

1. Urate synthesis and other metabolic characteristics of isolated chicken hepatocytes were studied. 2. The distinction is made between immediate precursors of the purine ring (glycine, glutamine, aspartate, formyltetrahydrofolate, bicarbonate) and ultimate precursors from which the immediate precursors are formed in the liver. 3. In hepatocytes from well-fed chickens the rate of urate synthesis was not greatly increased by the addition of amino acids or NH_4Cl , but in hepatocytes from 72 h-starved chickens the rate was much increased when alanine or asparagine was added as the only substrate. Other amino acids, when added alone, did not affect the rate. The exceptional effect of alanine and asparagine is due to the ready formation of the immediate precursors. 4. Conditions are described under which glutamine, serine, glycine plus formate, ribose and glucose increased the rate of urate synthesis. 5. At 1 mM- NH_4Cl (a concentration not much higher than that of blood plasma) the rate of urate synthesis in the presence of lactate was increased, but higher concentrations inhibited urate synthesis in the presence of lactate or alanine; with alanine even 1 mM- NH_4Cl was inhibitory. 6. Glucose synthesis from lactate, alanine or dihydroxyacetone was also inhibited by 1 mM- NH_4Cl . 7. NH_4Cl inhibition of urate and glucose synthesis was paralleled by an increased rate of glutamine synthesis. Thus in the presence of NH_4Cl the gluconeogenic precursors are diverted from the pathway of gluconeogenesis to that of glutamate and glutamine synthesis. This implies that the synthesis of these amino acids is the primary process in the detoxication of ammonia in the avian liver. 8. Urate synthesis, like urea synthesis, can be looked on as a cyclic process with either phosphoribosyl pyrophosphate or ribose acting as the carrier on which the purine ring is assembled. 9. The energy requirements of urate synthesis depend on whether phosphoribosyl pyrophosphate is regenerated from IMP by pyrophosphorylase or by phosphorylation and pyrophosphorylation of ribose. It is 6 or 9 pyrophosphate bonds of ATP respectively.

The starting point of this work was the intention to use isolated hepatocytes from chicken liver for the study of the rate-limiting factor in uric acid (i.e. purine ring) synthesis. The immediate precursors, as well as the ultimate precursors, of the purine ring are known (see Tables 1 and 2), but information on the factors that determine the rate of the purine-ring synthesis in the intact cell is limited. Barratt *et al.* (1974) found in the perfused chicken liver that the synthesis of uric acid was only marginally stimulated by the addition of the immediate precursors, i.e. glycine, glutamine, aspartate or NH_4Cl . In the present experiments on isolated hepatocytes the addition of the immediate precursors listed in Table 1 gave substantial increases in the rates of uric acid synthesis. Unexpectedly, the addition of NH_4Cl above 1 mM strongly inhibited purine-ring synthesis

and also gluconeogenesis, by promoting the synthesis of glutamine and thereby diverting carbon away from both the formation of the immediate precursors for urate and from gluconeogenesis. Thus the primary reaction disposing of excess of ammonia and controlling the concentration of ammonia in chicken liver is the synthesis of glutamine, with glutamate as an intermediate.

Materials and Methods

Chicks

Male Rhode Island Red $\times$ Light Sussex chicks (5–7 weeks old) were obtained from Orchards Farm, Great Missenden, Bucks., U.K., and were fed on Chick Starter Crumbs ACS, BOCM Silcock, Basingstoke, Hants., U.K. Before the preparation of the cells the birds had been starved for 48 or 72 h because this increased the rate of urate synthesis.

* Present address: Texas A and M University, and Department of Biochemistry and Biophysics, College Station, TX 77843, U.S.A.

Preparation of isolated liver cells and incubation conditions

Preparation of the cells was essentially by the method of Berry & Friend (1969) as described by Cornell *et al.* (1973) and Krebs *et al.* (1974). Because of the absence of a diaphragm in the avian body, a modification to the cannulation technique was necessary. To minimize the anoxic period between the opening of the body cavity and the start of the perfusion, a cannula was stuck through the heart into the inferior vena cava, and tied so that it rested just proximal to the entry of the hepatic vein. The perfusion of oxygenated medium was begun immediately after the insertion of the cannula, and the portal vein was cut to allow the perfusion medium to flow through the liver. The gizzard and intestines were then removed to form a cavity into which the perfusion medium drained. The first 90 ml of the perfused medium was discarded. After this 110 ml of perfusion medium containing 20 mg of collagenase was recirculated through the liver for 15 min. The liver outflow was returned to the reservoir by a roller pump. Söling *et al.* (1973) and Barratt *et al.* (1974) perfused the avian liver by the portal vein. Perfusion in the reverse direction was preferred because it made it possible to shorten the anoxic period of the liver. The perfusion of oxygenated medium began within 2 min of opening the body cavity. This does not imply that the liver had been anoxic for 2 min, because the vessels contained circulating blood; the heart was beating until the insertion of the cannula.

Cell suspensions were handled in the same manner as described by Krebs *et al.* (1974) including the measurement of O_2 consumption, with about 50 mg wet wt. of cells per 4 ml. The rates of all the metabolic

processes tested were approximately linear with time for 1 h, provided that the added substrates were not exhausted before the end of the hour.

Analytical methods

Lactate, pyruvate, 3-hydroxybutyrate, acetoacetate, glucose, glutamate, ammonia and alanine were measured on KOH-neutralized $HClO_4$ extracts by standard enzymic methods as described by Bergmeyer (1970). Uric acid was determined by the u.v. method of Scheibe *et al.* (1970) on samples that had been acidified with $HClO_4$ to stop metabolism, and that, without removal of the protein, were neutralized with KOH. Any insoluble material was then removed by centrifugation (2000g for 5 min). Radioactivity of urate was assayed by precipitating the urate with mercuric acetate (Bergmann & Dikstein, 1954), and dissolving the precipitate in 0.15 ml of ethanolamine, followed by the addition of 0.45 ml of methanol (Barratt *et al.*, 1974). The radioactivity of the total sample was counted in 10 ml of scintillation fluid, consisting of 5.5 g of Permablend II Packard (Packard Instrument Co., Caversham, Berks. RG4 7AA, U.K.), 60 g of naphthalene, 400 ml of 2-methoxyethanol and 600 ml of toluene.

Reagents

Enzymes and coenzymes were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Other chemicals were reagent grade. Radioactive compounds were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Rates of uric acid synthesis on addition of various substrates

The addition of single amino acids or mixtures of amino acids to isolated hepatocytes gave significant increases in the rate of urate synthesis in cells of 72 h-starved chickens (Table 3), but the effects in cells from well-fed chickens were much smaller (results not shown in Table 3). The highest rates on addition of single substrates were observed in the presence of either alanine or asparagine. Other amino acids, including serine and glycine (see Table 3), did not increase the endogenous rate when added

Table 1. *Immediate precursors of the purine ring in avian liver*

Data for the purine-ring precursors are from several sources (Buchanan & Sonne, 1946; Sonne *et al.*, 1946, 1948; Buchanan *et al.*, 1948).

Precursor	Contribution to purine ring
Glycine	$C_{(4)}$, $C_{(5)}$, $N_{(7)}$
Glutamine	$N_{(3)}$, $N_{(9)}$
Aspartate	$N_{(1)}$
Bicarbonate	$C_{(6)}$
Formyltetrahydrofolate	$C_{(2)}$, $C_{(8)}$

Table 2. *Ultimate precursors providing immediate precursors of the purine ring in avian liver*

Ultimate precursor	Immediate precursor
Proteolysis plus tetrahydrofolate	Glycine, glutamate, glutamine, aspartate, formyltetrahydrofolate
Glucose or gluconeogenic precursors plus NH_4^+ and tetrahydrofolate	Glutamate, aspartate, glutamine, glycine, formyltetrahydrofolate
Asparagine, or alanine plus tetrahydrofolate	Glutamate, aspartate, glutamine, glycine, formyltetrahydrofolate

Table 3. *Rates of urate synthesis on addition of various amino acids and other substrates*

Hepatocytes from 72h-starved chickens were incubated for 60min at 37°C. Initial substrate concentrations were 10mM, except for glutamine, which was 2mM. The data are means $\pm$ s.e.m. with numbers of tests in parentheses. Student's *t* test was applied to paired samples. A *P* = 0.05 was considered significant, indicated by s; ns, not significant.

Added substrates	Rate of urate synthesis (μ mol/min per g wet wt.)	Statistics
None	0.06 $\pm$ 0.02 (9)	—
Alanine	0.39 $\pm$ 0.04 (11)	—
Asparagine	0.32 $\pm$ 0.06 (4)	—
Glutamine	0.08 $\pm$ 0.01 (3)	—
Serine	0.08 $\pm$ 0.02 (3)	—
Glycine	0.07 $\pm$ 0.03 (3)	—
Alanine; glutamine	0.51 $\pm$ 0.05 (11)	s versus Ala
Alanine; glutamine; formate	0.28 $\pm$ 0.03 (3)	s versus Ala; Gln
Alanine; glutamine; glycine	0.36 $\pm$ 0.01 (3)	s versus Ala; Gln
Alanine; glutamine; glycine; formate	0.67 $\pm$ 0.05 (3)	s versus Ala; Gln
Alanine; glutamine; serine	0.54 $\pm$ 0.05 (3)	ns versus Ala; Gln
Alanine; glutamine; ribose; glucose	0.46 $\pm$ 0.01 (3)	ns versus Ala; Gln
Asparagine; glutamine	0.32 $\pm$ 0.04 (5)	ns versus Asn
Serine; glutamine	0.41 $\pm$ 0.06 (6)	s versus Ser
Serine; glutamine; formate	0.43 $\pm$ 0.07 (3)	ns versus Ser; Gln
Serine; glutamine; glycine	0.34 $\pm$ 0.05 (3)	s versus Ser; Gln
Serine; glutamine; glycine; formate	0.44 $\pm$ 0.06 (3)	ns versus Ser; Gln
Serine; glutamine; ribose	0.53 $\pm$ 0.07 (3)	s versus Ser; Gln
Serine; glutamine; glucose	0.47 $\pm$ 0.05 (3)	ns versus Ser; Gln
Serine; glutamine; ribose; glucose	0.50 $\pm$ 0.05 (3)	ns versus Ser; Gln
Serine; glutamine; ribose; glucose; formate	0.58 $\pm$ 0.01 (3)	s versus Ser; Gln s versus Ser; Gln; ribose; glucose
Glycine; formate	0.06 $\pm$ 0.03 (3)	ns versus Gly
Glycine; formate; glutamine	0.39 $\pm$ 0.09 (3)	s versus Gly; formate; Gln
Glycine; formate; glutamine; ribose	0.46 $\pm$ 0.09 (3)	s versus Gly; formate; Gln
Glycine; formate; glutamine; glucose	0.44 $\pm$ 0.07 (3)	s versus Gly; formate; Gln
Glycine; formate; glutamine; ribose; glucose	0.54 $\pm$ 0.10 (3)	s versus Gly; formate; Gln

alone. The exceptional behaviour of alanine and asparagine is not unexpected, as these are the only amino acids that readily form the immediate precursors listed in Table 1. Although these precursors can also be formed from glutamine and from serine or glycine, the pathways involve more steps and are not as rapid as those from alanine and asparagine.

Addition of glutamine caused major increases in the rate when either serine or glycine plus formate were also present. Glutamine caused a small increase in the presence of alanine, and when glutamine and alanine were present together the addition of glycine plus formate gave a significant increase, but there was no effect when glycine or formate or serine was added alone with glutamine and alanine. This implies that in the presence of glutamine and alanine the formation of glycine and formyltetrahydrofolate is a rate-limiting step. The addition of ribose or glucose gave rise to small but significant increases in the rate when serine plus glutamine or serine plus formate plus glutamine were present. Although ribose nucleotides and nucleosides are

intermediate stages in urate synthesis the ribose moiety is eventually released again so that it can act catalytically. Hence only small amounts of ribose are required and this may explain why the addition of ribose or its precursor glucose have only a slight effect.

The highest rates of urate synthesis were much lower in terms of ammonia detoxicated than those in rat liver (2.5 μ mol of NH_3 /min per g removed compared with 10 μ mol of NH_3 in rat liver). The rates in the present experiments were considerably higher than those found with chicken liver slices (Benzinger & Krebs, 1933), but of the same order as the rate of hypoxanthine synthesis under optimum conditions in pigeon liver (Örström *et al.*, 1939) and about twice as high as that observed in perfused chicken liver (Barratt *et al.*, 1974).

The extent to which added precursors contribute to the synthesis of urate was measured with [^{14}C]-alanine and with [^{14}C]asparagine, which supply carbons 2, 4, 5 and 8. From the data in Table 4 the specific radioactivity of these carbon atoms was

72% of ^{14}C from alanine and 97% of ^{14}C from asparagine when added alone. The greater loss of radioactivity from alanine can be explained by the dilution occurring at the stage of the pyruvate carboxylase reaction in the course of the conversion of ^{14}C from alanine into glycine and methylene-

tetrahydrofolate. The addition of unlabelled glutamine with [^{14}C]alanine or [^{14}C]asparagine decreased the ^{14}C label in urate to 52 and 81% respectively. Addition of unlabelled serine caused a further decrease in the incorporation of [^{14}C]alanine. The dilution of the urate ^{14}C label by glutamine and by serine indicates that these substances can also provide the immediate precursors.

Table 4. *Incorporation of [^{14}C]alanine and [^{14}C]asparagine into urate*

Hepatocytes from 72 h-starved chicks were incubated for 60 min at 37°C . The specific radioactivities of the [^{14}C]alanine and [^{14}C]asparagine were 5.40 and 3.15 d.p.m./mg-atom of carbon respectively. Initial substrate concentrations were 10 mM, except for glutamine, which was 2 mM. The data are means $\pm$ S.E.M. with numbers of observations in parentheses. * $P < 0.05$ by Student's t test when compared with the control. In the calculation of the specific radioactivity of ^{14}C in urate the radioactivity per molecule of urate was divided by 4 on the assumption that only carbon atoms 2, 4, 5 and 8 were derived from the labelled precursor, carbon-6 coming from the CO_2 pool.

Additions	Specific radioactivity of urate (d.p.m./mg-atom of carbon)
[^{14}C]Alanine	3.93 ± 0.10 (5)
[^{14}C]Alanine; glutamine	$2.88 \pm 0.15^*$ (5)
[^{14}C]Alanine; glutamine; serine	$1.36 \pm 0.09^*$ (3)
[^{14}C]Asparagine	3.00 ± 0.10 (5)
[^{14}C]Asparagine; glutamine	$2.48 \pm 0.13^*$ (3)

Effect of NH_4Cl on chicken liver metabolism

When NH_4Cl was the only added nitrogenous precursor the rates of urate synthesis were lower than in the presence of alanine or asparagine, with either lactate or dihydroxyacetone as carbon source (Table 5). In the presence of lactate the rates of urate synthesis were lower at 5 mM- and 10 mM- NH_4Cl than at 1 mM. In the presence of alanine even 1 mM- NH_4Cl was inhibitory. NH_4Cl at 1 mM or higher concentrations also inhibited gluconeogenesis from lactate and increased the accumulation of glutamine and (to a lesser extent) of glutamate (Table 6). The lactate carbon removed accounted for as glucose, glutamine, glutamate and pyruvate was the same both in the presence and in the absence of NH_4Cl . Thus NH_4Cl diverted lactate metabolism from glucose to glutamine synthesis.

As the acetyl-CoA required for the synthesis of glutamate and glutamine from lactate (via pyruvate, oxaloacetate, citrate and α -oxoglutarate) in all probability is derived from lactate, the flux through pyruvate dehydrogenase must have been accelerated

Table 5. *Effects of NH_4Cl on the rate of urate synthesis*

Hepatocytes prepared from 48 h-starved chicks were incubated for 1 h at 37°C . Initial substrate concentrations were 10 mM, except for glutamine, which was 2 mM, and for NH_4Cl , which are indicated in parentheses. The data are means $\pm$ S.E.M. for three animals or for one representative animal when no S.E.M. is given.

Substrates added	Urate synthesis ($\mu\text{mol/min per g wet wt.}$)
Lactate	0.05 ± 0.01
Lactate; NH_4Cl (1 mM)	0.30 ± 0.02
Lactate; NH_4Cl (5 mM)	0.24 ± 0.03
Lactate; NH_4Cl (10 mM)	0.18 ± 0.02
Dihydroxyacetone	0.04 ± 0.01
Dihydroxyacetone; NH_4Cl (1 mM)	0.32 ± 0.01
Dihydroxyacetone; NH_4Cl (5 mM)	0.36 ± 0.01
Dihydroxyacetone; NH_4Cl (10 mM)	0.32 ± 0.03
Alanine	0.38 ± 0.02
Alanine; NH_4Cl (1 mM)	0.30 ± 0.03
Alanine; NH_4Cl (5 mM)	0.20 ± 0.02
Alanine; NH_4Cl (10 mM)	0.20 ± 0.02
Alanine; glutamine; aspartate; glycine; formate	0.51
Alanine; glutamine; aspartate; glycine; formate; NH_4Cl (2 mM)	0.30
Glycine; formate; glutamine; glucose; ribose; NH_4Cl (0.2 mM)	0.53
Glycine; formate; glutamine; glucose; ribose; NH_4Cl (1.0 mM)	0.37
Glycine; formate; glutamine; glucose; ribose; NH_4Cl (2.0 mM)	0.31

Table 6. *Fate of lactate in the presence of NH₄Cl in chicken hepatocytes*

The animals were starved for 48 h. The concentration of cells was 52.4 ± 24 mg wet wt. per flask. The initial concentration of lactate was 11 mM and that of NH₄Cl as indicated. The values are μ mol per flask in 60 min (means $\pm$ S.E.M. for four different cell preparations). Values that are significantly different from the lactate control by the paired Student's *t* test ($P < 0.05$) are indicated by *. The sum of glucose, glutamate and glutamine formed from lactate was calculated on the assumption that two molecules of lactate are needed for each of these products.

	Lactate	Lactate; NH ₄ Cl (1 mM)	Lactate; NH ₄ Cl (5 mM)	Lactate; NH ₄ Cl (10 mM)
Lactate used	39 $\pm$ 0.6	38 $\pm$ 0.8	38 $\pm$ 0.8	38 $\pm$ 0.7
Pyruvate formed	0.20 $\pm$ 0.09	0.21 $\pm$ 0.08	0.18 $\pm$ 0.06	0.15 $\pm$ 0.03
Glucose formed	15 $\pm$ 0.7	13 $\pm$ 0.8*	10 $\pm$ 0.5*	9.0 $\pm$ 0.2*
Glutamine formed	-0.003 $\pm$ 0.003	0.37 $\pm$ 0.03*	4.6 $\pm$ 1.0*	4.7 $\pm$ 0.5*
Glutamate formed	0.02 $\pm$ 0.003	0.18 $\pm$ 0.01*	0.56 $\pm$ 0.05*	0.59 $\pm$ 0.03*
Glucose + glutamine + glutamate (as C ₃) formed	31	28	31	29
NH ₄ Cl used	-0.15 $\pm$ 0.04	3.6 $\pm$ 0.04*	11 $\pm$ 0.8*	14 $\pm$ 0.4*
Urate formed	0.15 $\pm$ 0.03	0.89 $\pm$ 0.06*	0.71 $\pm$ 0.09*	0.49 $\pm$ 0.05*
Alanine formed	0.03 $\pm$ 0.004	0.07 $\pm$ 0.01*	0.21 $\pm$ 0.02*	0.24 $\pm$ 0.01*

Table 7. *Metabolite changes on addition of lactate, dichloroacetate and NH₄Cl in chicken hepatocytes*

The animals were starved for 48 h. The concentration of cells was 52.4 ± 24 mg wet wt. per flask. The initial concentration of lactate was 11 mM, that of dichloroacetate 2 mM and that of NH₄Cl as indicated. The values are μ mol/flask in 60 min (means $\pm$ S.E.M. for four different cell preparations). Values that are significantly different from lactate; dichloroacetate control by the paired Student's *t* test ($P < 0.05$) are indicated by *. The sum of glucose, glutamate and glutamine formed from lactate was calculated on the assumption that two molecules of lactate are needed for each of these products.

	Lactate; dichloroacetate	Lactate; dichloroacetate; NH ₄ Cl (1 mM)	Lactate; dichloroacetate; NH ₄ Cl (5 mM)	Lactate; dichloroacetate; NH ₄ Cl (10 mM)
Lactate used	37 $\pm$ 1.0	37 $\pm$ 0.9	32 $\pm$ 1.8*	29 $\pm$ 1.6*
Pyruvate formed	0.08 $\pm$ 0.02	0.12 $\pm$ 0.04	0.18 $\pm$ 0.04	0.11 $\pm$ 0.02
Glucose formed	13 $\pm$ 1.1	12 $\pm$ 0.8	4.3 $\pm$ 0.5*	2.8 $\pm$ 0.43*
Glutamine formed	0.080 $\pm$ 0.04	0.80 $\pm$ 0.03*	6.5 $\pm$ 0.2*	6.9 $\pm$ 0.3*
Glutamate formed	0.04 $\pm$ 0.004	0.24 $\pm$ 0.01*	0.52 $\pm$ 0.07*	0.49 $\pm$ 0.05*
Glucose + glutamine + glutamate (as C ₃) formed	27	25	23	20
NH ₄ Cl used	-0.14 $\pm$ 0.09	3.5 $\pm$ 0.03*	16 $\pm$ 0.4*	16 $\pm$ 1.0*
Urate formed	0.23 $\pm$ 0.04	0.65 $\pm$ 0.02*	0.38 $\pm$ 0.06*	0.29 $\pm$ 0.05*
Alanine formed	0.03 $\pm$ 0.01	0.02 $\pm$ 0.01	0.08 $\pm$ 0.01*	0.09 $\pm$ 0.006*

by NH₄⁺. An activation of pyruvate dehydrogenase by NH₄⁺ has been demonstrated for rat liver by Häussinger *et al.* (1975).

Dichloroacetate, which activates pyruvate dehydrogenase (Whitehouse & Randle, 1973), increased glutamine synthesis and brought about a further inhibition of gluconeogenesis in the presence of NH₄Cl (Table 7). Thus dichloroacetate diverted more pyruvate from carboxylation to dehydrogenation. Again, the lactate carbon atoms removed, accounted for as glucose, glutamine, glutamate and pyruvate, were about the same in the absence and in the presence of NH₄Cl.

With alanine as the substrate, gluconeogenesis was inhibited already by 1 mM-NH₄Cl, whereas the synthesis of glutamine was increased (Table 8). The decrease in glucose formation was matched by an increase in the yields of glutamate and glutamine so that the sum of glucose plus glutamate plus glutamine remained approximately constant.

In the presence of dihydroxyacetone gluconeogenesis was inhibited by 1 mM-, 5 mM- or 10 mM-NH₄Cl (Table 9). The fact, already mentioned, that urate synthesis was not inhibited by NH₄Cl, in contrast with the inhibition that occurred in the presence of lactate and alanine, may be understood on the basis of the following considerations. The inhibition of urate synthesis by NH₄Cl in the presence of lactate or alanine, as discussed, is a matter of diversion of carbon atoms from the pathway leading to the formation of serine (and hence glycine and formyl-tetrahydrofolate) to the synthesis of α -oxoglutarate. With dihydroxyacetone this diversion would not interfere with the formation of serine because phosphoglycerate, the precursor of serine, is an intermediate in the pathway for the formation of glutamine via oxaloacetate. With lactate and alanine the diversion from the pathway of serine formation branches at the stage of oxaloacetate in favour of the synthesis of α -oxoglutarate, and does

Table 8. *Fate of alanine in the presence of NH₄Cl in chicken hepatocytes*

The animals were starved for 48 h. The concentration of cells was 50.5 ± 0.9 mg wet wt. per flask. The initial concentration of alanine was 10 mM and that of NH₄Cl as indicated. The values are μ mol per flask in 60 min (means $\pm$ S.E.M. for three different cell preparations). Values that are significantly different from the alanine control by the paired Student's *t* test ($P < 0.05$) are indicated by *.

	Alanine	Alanine; NH ₄ Cl (1 mM)	Alanine; NH ₄ Cl (5 mM)	Alanine; NH ₄ Cl (10 mM)
Alanine used	6.3 ± 0.2	4.7 ± 1.0	4.5 ± 0.7	4.2 ± 1.00
Glucose formed	1.2 ± 0.04	$0.39 \pm 0.03^*$	$0.22 \pm 0.05^*$	$0.14 \pm 0.02^*$
Glutamine formed	0.24 ± 0.15	$1.3 \pm 0.1^*$	$1.4 \pm 0.2^*$	$1.3 \pm 0.13^*$
Glutamate formed	0.23 ± 0.01	0.23 ± 0.01	0.21 ± 0.02	0.19 ± 0.01
Glucose + glutamine + glutamate (as C ₃) formed	3.4	3.8	3.6	3.2
NH ₄ Cl used	0.15 ± 0.10	2.4 ± 0.2	2.3 ± 0.5	2.6 ± 0.2
Urate formed	1.1 ± 0.1	$0.90 \pm 0.08^*$	$0.62 \pm 0.06^*$	$0.60 \pm 0.07^*$

Table 9. *Fate of dihydroxyacetone in the presence of NH₄Cl in chicken hepatocytes*

The animals were starved for 48 h. The concentration of cells was 52.4 ± 24 mg wet wt. per flask. The initial concentration of dihydroxyacetone was 10 mM and that of NH₄Cl as indicated. In calculating the sum of dihydroxyacetone converted into glucose, glutamine and glutamate it was assumed that two molecules of dihydroxyacetone are needed for one molecule of glucose and one molecule of the amino acids. The values are changes in μ mol per flask in 60 min (means $\pm$ S.E.M. for four different cell preparations). Values that are significantly different from those obtained in the absence of NH₄Cl by the paired Student's *t* test ($P < 0.05$) are indicated by *.

	Dihydroxyacetone	Dihydroxyacetone; NH ₄ Cl (1 mM)	Dihydroxyacetone; NH ₄ Cl (5 mM)	Dihydroxyacetone; NH ₄ Cl (10 mM)
Glucose formed	10.2 ± 0.5	$8.4 \pm 0.1^*$	$7.3 \pm 0.4^*$	$7.1 \pm 0.7^*$
Glutamine formed	0.09 ± 0.03	0.16 ± 0.03	$0.92 \pm 0.02^*$	$1.11 \pm 0.11^*$
Glutamate formed	0.02 ± 0.01	$0.09 \pm 0.01^*$	$0.19 \pm 0.01^*$	$0.22 \pm 0.03^*$
Glucose + glutamine + glutamate (as C ₃) formed	10.3	8.6	8.4	8.4
NH ₄ Cl used	-0.15 ± 0.06	3.5 ± 0.1	6.1 ± 0.4	7.6 ± 0.8
Urate formed	0.13 ± 0.04	$0.97 \pm 0.07^*$	$1.09 \pm 0.04^*$	$0.96 \pm 0.10^*$
Lactate + pyruvate formed	0.19 ± 0.03	0.20 ± 0.04	0.20 ± 0.02	0.20 ± 0.04
Alanine formed	0.022 ± 0.004	0.022 ± 0.005	0.012 ± 0.006	0.009 ± 0.003

not leave sufficient oxaloacetate for the formation of phosphopyruvate, phosphoglycerate and serine.

Effect of acetate and other fatty acids on urate synthesis

If hypoxanthine is formed from IMP by the pyrophosphorylase reaction (see the Discussion section, reaction 1) the intracellular concentration and supply of pyrophosphate might be expected to be a rate-limiting factor. To test this, acetate and other fatty acids were added because the conversion of fatty acids into acyl-CoA is accompanied by a stoichiometric formation of pyrophosphate. The experiments (Table 10) show that neither acetate nor other fatty acids caused a significant increase in urate synthesis with alanine as the nitrogen donor. The experiments also show that in chicken hepatocytes the rate of ketone-body formation from acetate was much higher than in rat hepatocytes, and as high as that from oleate or butyrate. This implies that the rate of pyrophosphate formation was very much higher in the presence of acetate compared with the other fatty acids.

In liver from starved rats the increase in ketone-body formation on addition of acetate is hardly significant in relation to the control value of about 0.5μ mol/min per g wet wt. (J. Medina, unpublished work; see also Cederbaum & Rubin, 1975). In chicken hepatocytes the rate in the control without added substrate was 0.76μ mol/min per g and acetate raised this rate to 2.6. The ratio [β -hydroxybutyrate]/[acetoacetate] was highly variable in chicken hepatocytes, possibly because of a relatively low activity of β -hydroxybutyrate dehydrogenase. Attempts to assay this enzyme in chicken liver proved unsatisfactory: the method of Lehninger *et al.* (1960), which gives satisfactory results on rat tissue, gave values much lower than expected from the rate of formation of β -hydroxybutyrate in the presence of oleate or of acetoacetate plus lactate in intact cells.

Acetate added alone had no effect on the O₂ uptake, although the synthesis of ketone bodies from acetate must have consumed much of the available ATP. Addition of acetate plus alanine caused a trebling of the O₂ uptake, which was not accompanied by an increase in the energy-requiring processes (the

Table 10. *Effect of acetate and other fatty acids on urate synthesis, ketone-body formation and O₂ consumption in hepatocytes from 48 h-starved chickens*. Hepatocytes were incubated for 50 min at 37°C. The initial concentrations of added substrates were 10 mM-alanine, 15 mM-acetate, 10 mM-butyrate and 1 mM-oleate. The values are $\mu\text{mol/min per g wet wt.}$ except for the ketone-body ratios (means $\pm$ S.E.M. for three cell preparations). Ketone-body ratios were calculated on the basis of the ketone-body concentrations found at 50 min and not on the basis of ketone bodies formed during incubation.

Substrates added	O ₂ used	Urate formed	3-Hydroxybutyrate formed	Acetoacetate formed	Total ketone bodies formed	[3-Hydroxybutyrate] [acetoacetate]	Glucose formed	Glutamate formed	Glutamine formed
None	2.0 $\pm$ 0.1	0.07 $\pm$ 0.01	0.42 $\pm$ 0.04	0.34 $\pm$ 0.96	0.76 $\pm$ 0.04	1.5 $\pm$ 0.5	0.11 $\pm$ 0.02	0.006 $\pm$ 0.002	Nil
Alanine	3.8 $\pm$ 0.3	0.47 $\pm$ 0.01	0.33 $\pm$ 0.04	-0.018 $\pm$ 0.008	0.31 $\pm$ 0.03	9.0 $\pm$ 0.6	0.75 $\pm$ 0.10	0.069 $\pm$ 0.002	0.37 $\pm$ 0.12
Alanine; acetate	6.0 $\pm$ 0.9	0.55 $\pm$ 0.06	2.5 $\pm$ 0.3	0.69 $\pm$ 0.33	3.2 $\pm$ 0.5	5.6 $\pm$ 2.2	0.55 $\pm$ 0.14	0.11 $\pm$ 0.03	0.38 $\pm$ 0.13
Alanine; butyrate	5.6 $\pm$ 1.0	0.47 $\pm$ 0.04	2.0 $\pm$ 0.2	0.67 $\pm$ 0.18	2.7 $\pm$ 0.4	3.6 $\pm$ 1.1	0.66 $\pm$ 0.11	0.21 $\pm$ 0.06	0.34 $\pm$ 0.11
Alanine; oleate	4.9 $\pm$ 0.4	0.29 $\pm$ 0.03	2.0 $\pm$ 0.3	0.23 $\pm$ 0.13	2.2 $\pm$ 0.2	14 $\pm$ 6	0.39 $\pm$ 0.02	0.23 $\pm$ 0.06	0.15 $\pm$ 0.05
Acetate	2.1 $\pm$ 0.4	0.067 $\pm$ 0.008	0.73 $\pm$ 0.06	1.8 $\pm$ 0.3	2.6 $\pm$ 0.4	0.45 $\pm$ 0.07	0.075 $\pm$ 0.016	Nil	Nil
Butyrate	3.8 $\pm$ 0.4	0.064 $\pm$ 0.002	0.98 $\pm$ 0.15	2.0 $\pm$ 0.3	3.0 $\pm$ 0.3	0.53 $\pm$ 0.11	0.099 $\pm$ 0.024	Nil	Nil
Oleate	3.6 $\pm$ 0.2	0.085 $\pm$ 0.012	1.94 $\pm$ 0.09	0.42 $\pm$ 0.07	2.4 $\pm$ 0.1	3.2 $\pm$ 1.0	0.083 $\pm$ 0.016	Nil	Nil

synthesis of glucose, glutamate and glutamine) (Table 10). No satisfactory explanation can be offered for this apparent discrepancy.

O₂ consumption in relation to energy requirements

When lactate was the only substrate the rate of O₂ consumption increased about 3-fold over the control, and the extra ATP supplied by the additional O₂ consumption agreed very closely with the extra ATP required on the basis of the assumptions defined in the legend of Table 11. This agreement between maximum efficiency of oxidative phosphorylation and of ATP utilization shows that substrate cycling was minimal. In the isolated perfused rat liver and pigeon liver the efficiency of glucose synthesis from lactate is below the calculated minimum (Hems *et al.*, 1966; Williamson *et al.*, 1969; Söling *et al.*, 1973). It is also lower in rat kidney-cortex slices (Krebs *et al.*, 1963).

When lactate together with NH₄Cl were the substrates the rate of extra ATP formation was somewhat higher than expected. As the amount of urate formed was small no significant error would arise if the ATP requirement of urate synthesis were 9 rather than 6 ATP per molecule. In the perfused rat liver the addition of a nitrogenous substrate (NH₄Cl) also decreases the efficiency of ATP utilization (Hems *et al.*, 1966).

Discussion

Supply of immediate precursors of the purine ring

The effects of added substances on the rate of urate synthesis were on the whole as expected on the basis of the existing knowledge of the pathway of purine synthesis. It is one of the functions of the avian liver to produce the immediate precursors from the ultimate precursors supplied by the portal blood and the hepatic arteries. When 4 molecules of NH₃ are converted into urate 1 molecule of glycine, 2 molecules of glutamine, 1 molecule of aspartate and 2 molecules of formyltetrahydrofolate are required. The amino acid mixture reaching the liver, plus the hepatic formation of glycine and formyltetrahydrofolate from serine and from histidine, does not supply sufficient amounts of glycine, glutamine and tetrahydrofolate derivatives. These substances must therefore be synthesized by the liver. The ready synthesis of glutamine from pyruvate in avian liver, with 2-oxoglutarate as an intermediate, has long been known (Örström *et al.*, 1939; Evans, 1940), the rate being three to five times higher in avian than in rat liver. When this was discovered the significance of glutamine synthesis in avian liver was not understood. It is now evident that it is an essential step in the synthesis of urate.

Similarly the relevance to purine synthesis of the high capacity of the pathway from carbohydrate

Table 11. *O₂ consumption, urate synthesis and gluconeogenesis in the presence of lactate or lactate plus NH₄Cl*

Hepatocytes from 48 h-starved chickens were incubated for 50 min at 37°C. The initial concentrations were 10 mM-lactate and 5 mM-NH₄Cl. The values are $\mu\text{mol/min per g wet wt.}$ (means $\pm$ S.E.M. for three cell preparations). The calculations were based on the assumption that synthesis requirements per molecule were as follows: glucose, 6 ATP; glutamate, 1 ATP; glutamine, 2 ATP; urate, 6 ATP; further, that on average the consumption of 1 molecule of O₂ yielded 6 ATP. The O₂ used in the conversion of hypoxanthine into urate, which is probably not coupled to phosphorylation, was negligible.

	Substrates added ...	None	Lactate	Lactate; NH ₄ Cl
Metabolic changes				
O ₂ used		2.0 $\pm$ 0.1	6.7 $\pm$ 0.6	7.0 $\pm$ 0.8
Glucose formed		0.11 $\pm$ 0.02	5.0 $\pm$ 0.6	3.4 $\pm$ 0.4
Glutamate formed		Nil	0.02 $\pm$ 0.01	0.12 $\pm$ 0.01
Glutamine formed		Nil	Nil	1.0 $\pm$ 0.1
Urate formed		0.07 $\pm$ 0.01	0.064 $\pm$ 0.007	0.32 $\pm$ 0.04
Calculations				
Rate of ATP required for:				
Glucose formation		0.66	30.0	20.4
Glutamate formation		—	—	0.1
Glutamine formation		—	—	2.0
Urate formation		0.42	0.38	1.9
Total		1.08	30.4	24.4
Rate of ATP formation:				
Total		12.0	40.1	42.0
By extra O ₂ consumption			28.1	30.0

(3-phosphoglycerate) to serine in avian liver discovered by Willis & Sallach (1964) and Walsh & Sallach (1966) does not seem to have been appreciated by the discoverers.

The fact that alanine as the only added substrate promotes urate synthesis more effectively than any other single amino acid except asparagine is of interest in connection with the predominance of alanine (and glutamine) in the blood reaching the liver. In the mammalian body much of the nitrogen derived from the degradation of amino acids in the peripheral tissues is transported to the liver in the form of alanine and glutamine (Marliss *et al.*, 1971). In addition the portal blood supplies alanine formed from glutamate, glutamine and aspartate in the intestinal wall (Neptune, 1965; Windmueller & Spaeth, 1974). It is likely that this holds also for the avian organism. It is therefore significant that alanine, though it is not an immediate precursor of the purine ring, is an effective ultimate precursor.

Effect of NH₄Cl

The inhibitory effect of NH₄Cl both on urate synthesis and on glucose synthesis from lactate or alanine and the parallel acceleration of glutamine

synthesis indicate that regulatory mechanisms are so arranged that NH₃ detoxication takes precedence over other metabolic processes, especially gluconeogenesis and the provision of the carbon precursors of the purine ring. Thus oxaloacetate, instead of forming phosphoenolpyruvate, is used for the synthesis of excess of 2-oxoglutarate, which binds NH₃ by forming glutamate and glutamine. Dihydroxyacetone is also diverted from the aldolase reaction (after phosphorylation) to form pyruvate and eventually 2-oxoglutarate.

The implication is that the synthesis of glutamate and glutamine is the primary process in the detoxication of NH₃. The subsequent synthesis of urate and its excretion has two major physiological advantages. It involves the recovery of the carbon skeleton of glutamate and glutamine and thus saves sources of energy, and it converts the surplus nitrogen into a substance of low solubility that can be excreted almost as a solid, a matter of importance to birds, which in the interest of weight limitation in flying carry only limited amounts of water.

Urate synthesis as a cyclic process. The formation of hypoxanthine from IMP in the avian liver could be brought about by two different pathways:

Reaction (1) in the reverse direction is one of the purine salvage mechanisms. Its occurrence in the direction indicated would depend on the supply and steady-state concentration of the reactants, especially PP_i , and the equilibrium position of the reaction. The enzymes catalysing sequence (2) are widespread, but the occurrence of this sequence would involve a significant loss of energy. The occurrence of reaction (1) in chicken hepatocytes is supported by the finding that addition of acetate, which generates PP_i when metabolized, accelerates the rate of urate synthesis from alanine (Table 10).

The PP_i used in the synthesis of phosphoribosyl pyrophosphate from IMP is immediately regenerated in the next step of purine synthesis when phosphoribosyl pyrophosphate reacts with glutamine to form phosphoribosylamine. Thus provided that the PP_i is not hydrolysed it need not be continuously supplied from ATP as it acts in the manner of a catalyst.

If reaction (1) is the preferred mechanism of IMP degradation, one can look on phosphoribosyl pyrophosphate as the carrier on which the purine ring is assembled. As the carrier is regenerated by reaction (1) the pathway of urate synthesis could be formulated as a cycle, as illustrated in an abbreviated form by Scheme 1.

If the sequence (2) is the main pathway the carrier which is regenerated is ribose and the following reactions would have to be inserted into the cyclic sequence between IMP and phosphoribosyl pyrophosphate:

This would mean an extra consumption of 3-pyrophosphate bonds of ATP.

Energy requirements of urate synthesis

The ATP requirement depends on whether phosphoribosyl pyrophosphate in the urate cycle is

Scheme 1. *Urate synthesis as a cyclic process (abbreviated form)*

regenerated by reaction (1) or by reaction (2); it is 6 or 9 pyrophosphate bonds of ATP respectively. Thus for each nitrogen atom 1.5 or 2.25 pyrophosphate bonds of ATP are needed. Mammalian urea synthesis needs 2 pyrophosphate bonds per nitrogen atom.

There is, however, another aspect to be considered. The synthesis of each molecule of uric acid consumes 1 molecule of glycine and thereby prevents the combustion of a glycine equivalent. As the complete oxidation of glycine requires 3 oxygen atoms, the oxidizable material contained in urate corresponds to a maximum loss of 9 pyrophosphate bonds of ATP. The disadvantages arising from the loss of usable energy may be compensated by the advantage of having a nitrogenous end product that can be discharged without much loss of water.

Analogies between urea synthesis and the avian and mammalian livers

There are noteworthy analogies, relevant to biochemical evolution, between urea synthesis in the mammalian body and urate synthesis in the avian body. In both processes one-half of the nitrogen passes through the stage of NH_3 and one-half through the stage of amino nitrogen. In urea synthesis the amino N contributing to urea synthesis is that of glutamate and aspartate, in urate synthesis it is that of aspartate and glycine. In urea synthesis NH_3 is involved in the formation of carbamoyl phosphate, in urate synthesis in the formation of glutamine. Both processes involve glutamate dehydrogenase as one of the primary NH_3 -binding processes. Both are adaptations of processes that have arisen very early in evolution. Urea synthesis is a combination of the synthesis of arginine from ornithine with the action of arginase, two processes that occur already in many micro-organisms. Urate synthesis is a combination of a partial process of nucleic acid synthesis with xanthine oxidase. Finally, both processes represent cyclic mechanisms in which the nitrogenous end product is assembled on a carrier that is regenerated.

Xanthine oxidase and phosphoribosyl pyrophosphate amidotransferase as rate-limiting factors

The capacity of xanthine oxidase in chicken liver is subject to major adaptive influences; after starvation, it increases 4-fold compared with the value in the fed state (Della Corte & Stirpe, 1967). The maximum capacities are only slightly higher than the maximum rate of urate synthesis. This makes sense physiologically because IMP is not only an intermediate in urate formation but also a precursor of AMP and GMP. It is therefore essential that only the excess of IMP be removed by xanthine oxidase. Any increase in the concentration of hypoxanthine (caused by low activity of xanthine oxidase) would retard or stop the

degradation of IMP by the pyrophosphorylase reaction, because of the ready reversibility of this reaction, and preserve IMP for the synthesis of adenine and guanine nucleotides.

In non-uricotelic organisms, when purine synthesis serves only to supply purine nucleotides, synthesis is regulated mainly through feedback inhibition of phosphoribosyl pyrophosphate amidotransferase (EC 2.4.2.14) by adenine and guanine nucleotides (Wyngaarden & Ashton, 1959). Thus, when enough adenine and guanine nucleotides are present further synthesis of purine ceases. In uricotelic organisms there must be additional regulatory mechanisms because urate synthesis from excess of nitrogen must go on even when the cells have their full complement of nucleotides. According to Caskey *et al.* (1964) the controlling factor is the concentration of glutamine, the primary product of NH_3 detoxication: glutamine reverses the inhibition of phosphoribosyl pyrophosphate amidotransferase by nucleotides.

This work was supported by a postdoctoral Fellowship from the U.S. National Institutes of Health (Grant no. F32 CA 05057), from the National Cancer Institute (J. P. M.) and by grants from the Medical Research Council, U.K., and the U.S. Public Health Service (Grant no. 5 RO1 AM 11748-07 MET). We thank Mr. P. Gregory for preparation of the liver cells and Mr. D. Wiggins for technical assistance.

References

- Barratt, E., Buttery, P. J. & Boorman, K. N. (1974) *Biochem. J.* **144**, 189-198
- Benzinger, Th. & Krebs, H. A. (1933) *Klin. Wschr.* **12**, 1206-1208
- Bergmeyer, H. U. (ed.) (1970) *Methods of Enzymatic Analysis*, 2nd edn., Verlag Chemie, Weinheim
- Bergmann, F. & Dikstein, S. (1954) *J. Biol. Chem.* **211**, 149-153
- Berry, M. N. & Friend, D. S. (1969) *J. Cell Biol.* **43**, 506-520
- Buchanan, J. M. & Sonne, J. C. (1946) *J. Biol. Chem.* **166**, 781
- Buchanan, J. M., Sonne, J. C. & Delluva, A. M. (1948) *J. Biol. Chem.* **173**, 81-98
- Caskey, C. T., Ashton, D. M. & Wyngaarden, J. B. (1964) *J. Biol. Chem.* **239**, 2570-2579
- Cederbaum, A. I. & Rubin, E. (1975) *Arch. Biochem. Biophys.* **166**, 618-628
- Cornell, N. W., Lund, P., Hems, R. & Krebs, H. A. (1973) *Biochem. J.* **134**, 671-672
- Della Corte, E. & Stirpe, F. (1967) *Biochem. J.* **102**, 520-524
- Evans, E. A., Jr. (1940) *Biochem. J.* **34**, 829-837
- Häussinger, D., Weiss, L. & Sies, H. (1975) *Eur. J. Biochem.* **52**, 421-431
- Hems, R., Ross, B. D., Berry, M. N. & Krebs, H. A. (1966) *Biochem. J.* **101**, 281-292
- Krebs, H. A., Hems, R. & Gascoyne, T. (1963) *Acta Biol. Med. Germ.* **11**, 607-615

- Krebs, H. A., Cornell, N. W., Lund, P. & Hems, R. (1974) in *Alfred Benzon Symposium 6th* (Lundquist, F. & Tystrup, N., eds.), pp. 718–743, Munksgaard, Copenhagen
- Lehninger, A. L., Sudduth, H. C. & Wise, J. B. (1960) *J. Biol. Chem.* **235**, 2450–2455
- Marliss, E. B., Aoki, T. T., Pozefsky, P., Most, A. S. & Cahill, G. F. (1971) *J. Clin. Invest.* **50**, 814–817
- Neptune, E. M., Jr. (1965) *Am. J. Physiol.* **209**, 329–332
- Örström, A., Örström, M., Krebs, H. A. & Eggleston, L. V. (1939) *Biochem. J.* **33**, 995–999
- Scheibe, P., Bernt, E. & Bergmeyer, H. U. (1970) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), 2nd edn., pp. 1951–1954, Verlag Chemie, Weinheim
- Söling, H. D., Kleineke, J., Willms, B., Janson, G., Kuhn, A. (1973) *Eur. J. Biochem.* **37**, 233–243
- Sonne, J. C., Buchanan, J. M., Delluva, A. M. (1946) *J. Biol. Chem.* **166**, 395–396
- Sonne, J. C., Buchanan, J. M. & Delluva, A. M. (1948) *J. Biol. Chem.* **173**, 69–79
- Walsh, D. A. & Sallach, H. J. (1966) *J. Biol. Chem.* **241**, 4068–4076
- Whitehouse, S. & Randle, P. J. (1973) *Biochem. J.* **134**, 651–653
- Williamson, J. R., Scholz, R. & Browning, E. T. (1969) *J. Biol. Chem.* **244**, 4617–4627
- Willis, J. E. & Sallach, H. J. (1964) *Biochim. Biophys. Acta* **81**, 39–54
- Windmueller, H. G. & Spaeth, A. E. (1974) *J. Biol. Chem.* **249**, 5070–5079
- Wyngaarden, J. B. & Ashton, D. M. (1959) *J. Biol. Chem.* **234**, 1492–1496