CXXI. THE FORMATION OF HYPOXANTHINE IN PIGEON LIVER

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EDSON *et al.* [1936] isolated hypoxanthine from solutions in which pigeon liver had been allowed to metabolize ammonium salts. Reindel & Schuler [1937] on the other hand, using alanine as substrate, found only traces of hypoxanthine and claimed that xanthine is the chief purine base synthesized in pigeon liver. In order to elucidate this discrepancy we re-examined the nature of the purine base formed using the method described in the preceding paper. In our former experiments hypoxanthine was isolated as hypoxanthine-AgNO₃ but the yields were not quantitative owing to unavoidable losses during the isolation and purification. The isolation experiments therefore left it open whether some xanthine is formed in addition to hypoxanthine. The new method enabled us to determine both purine bases quantitatively. The experiments confirm our previous results: hypoxanthine is virtually the only purine base synthesized in pigeon liver. Although the experimental conditions were widely varied we were unable to find significant quantities of xanthine.

The second part of the paper deals with the rate of hypoxanthine synthesis under varying conditions. Oxaloacetate and glutamine were found to increase specifically the rate of hypoxanthine synthesis. These substances may therefore be assumed to play a special part in the hypoxanthine synthesis, the nature of which, however, remains to be investigated.

Methods

The experiments were carried out in conical manometric flasks. Slices of pigeon liver were shaken at 40° in a saline medium containing 0.025 *M* NaHCO₃-CO₂ or phosphate buffer. Various substrates were added in the form of 0.2 *M* neutral solutions. After 1 or 2 hr. the slices were removed from the solution. If NaHCO₃-saline was used, 0.2 ml. 0.5 *M* KH₂PO₄ was added to the solution and 0.2 ml. 2*N* NaOH was placed in the centre cup of the manometric flask. The flask was then shaken for 20 min. in the water bath in order to remove the CO₂ from the solution. The flask was again disconnected; 1.0 ml. 0.1 *M* phosphate buffer of pH 7.4 and 0.5 ml. liver extract were placed in the main compartment and 0.2 ml. 10% xanthine oxidase in the side-arm. If the medium was phosphate saline the addition of KH₂PO₄ and the subsequent shaking for 20 min. were omitted. The O₂ uptake and uric acid formation were determined as described in the preceding paper. A blank determination on the reagents was carried out in all experiments.

Added hypoxanthine and xanthine were quantitatively recovered under these conditions.

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Nature of the purine base synthesized in pigeon liver

Some experiments in which the substrates and media were varied are recorded in Table I. The ratio $\frac{\text{uric acid}}{\text{O}_2}$ (last column) is, within the limits of error, 1.0, as is expected for hypoxanthine. The deviations are insignificant and in both directions. This leaves no doubt that the bulk of the purine base formed in pigeon liver is hypoxanthine.

Table I. *Determination of the purine base formed in pigeon liver*

40°. Period of incubation 60 min.

Substrate (final conc.)	Gas	Tissue mg.	In presence of xanthine oxidase		Ratio uric acid O ₂
			$\mu\text{l. O}_2$ absorbed	$\mu\text{l. uric}$ acid formed	
NaHCO ₃ saline:					
—	5% CO ₂ in O ₂	27.7	24.9	24.0	0.96
l(+)Alanine (0.02 M)	"	24.8	31.3	28.7	0.95
l(+)Glutamic acid (0.02 M)	"	25.6	25.2	24.2	0.96
l(+)Glutamine (0.02 M)	"	26.0	50.8	52.6	1.04
l(−)Aspartic acid (0.02 M)	"	29.0	31.2	28.2	0.91
l(−)Asparagine (0.02 M)	"	28.9	39.2	42.8	1.09
0.003 M NH ₄ Cl; 0.02 M pyruvate	"	22.6	39.0	36.4	0.93
0.003 M NH ₄ Cl; 0.02 M oxaloacetate	"	27.1	53.1	55.9	1.05
0.02 M Oxaloacetate; 0.02 M l(+) glutamine	"	21.6	55.0	56.4	1.02
—		14.06	15.0	13.3	0.89
—	5% CO ₂ in N ₂	44.04	12.5	11.2	0.90
0.04 M Pyruvate; 0.02 M NH ₄ Cl	5% CO ₂ in O ₂	17.04	36.1	33.2	0.91
0.04 M Oxaloacetate; 0.02 M NH ₄ Cl	"	16.12	38.5	34.7	0.90
0.02 M Oxaloacetate; 0.01 M NH ₄ Cl	5% CO ₂ in N ₂	44.77	14.7	12.2	0.84
Phosphate saline:					
0.02 M Pyruvate; 0.01 M NH ₄ Cl	O ₂	14.90	13.7	13.0	0.95
0.02 M Oxaloacetate; 0.01 M NH ₄ Cl	"	9.57	23.5	22.8	0.97

Rate of hypoxanthine synthesis

Since hypoxanthine is the only purine base formed in pigeon liver, its concentration in the medium may be calculated directly from the amount of O₂ absorbed in the presence of xanthine oxidase. We have therefore dispensed with the determination of the uric acid formation in the further experiments which were carried out to measure the rate of hypoxanthine formation under varying conditions. Eleven sets of typical experiments are recorded in Table II. The rates are expressed by the quotient:

$$Q_{\text{hypoxanthine}} = \frac{\mu\text{l. O}_2 \text{ absorbed in the presence of xanthine oxidase}}{\text{mg. (dry) liver used} \times \text{hr. of incubation}}$$

These experiments show the following:

(1) There is a small formation of hypoxanthine when no substrate is added (last column). This "blank" is still much smaller under anaerobic conditions (Exp. 4). It is probable that the hypoxanthine formed anaerobically does not arise from synthesis but from the breakdown of adenine nucleotides.

(2) Addition of amino-acid causes an increase of the rate of hypoxanthine synthesis (Exps. 1, 9). Among the substances tested glutamine has the largest effect (Exps. 1, 2, 3, 4, 5, 11). The increase does not occur under anaerobic conditions.

(3) NH₄Cl causes an increase of the rate of synthesis if added in low concentrations (0.005 M). In higher concentrations it tends to inhibit (Exp. 5).

Table II. *Rate of hypoxanthine synthesis in pigeon liver at 40°*

No.	Gas	Substrates (final conc.)	Q hypoxanthine	
			With substrate	No substrate
		NaHCO ₃ saline		
1	5% CO ₂ in O ₂	0.02 <i>M</i> <i>l</i> (+)-Alanine	1.26	0.90
		0.02 <i>M</i> <i>l</i> (+)-Glutamine	1.95	0.90
		0.02 <i>M</i> <i>l</i> (+)-Glutamate	0.99	0.90
		0.02 <i>M</i> <i>l</i> (-)-Aspartate	1.07	0.90
		0.02 <i>M</i> <i>l</i> (-)-Asparagine	1.35	0.90
		0.02 <i>M</i> <i>l</i> (-)-Arginine	0.81	0.90
		0.0033 <i>M</i> NH ₄ Cl; 0.02 <i>M</i> oxaloacetate	1.97	0.90
		0.02 <i>M</i> <i>l</i> (+)-Glutamine; 0.02 <i>M</i> oxaloacetate	2.54	0.90
		0.0033 <i>M</i> NH ₄ Cl; 0.02 <i>M</i> pyruvate	1.72	0.90
2	"	0.01 <i>M</i> <i>l</i> (+)-Glutamine	1.79	1.03
3	"	0.01 <i>M</i> <i>l</i> (+)-Glutamine	1.31	0.71
		0.0066 <i>M</i> NH ₄ Cl; 0.02 <i>M</i> oxaloacetate	2.60	0.71
		" 0.02 <i>M</i> succinate	1.06	0.71
		" 0.02 <i>M</i> <i>l</i> (-)-malate	0.82	0.71
	"	" 0.02 <i>M</i> acetoacetate	0.92	0.71
4	5% CO ₂ in N ₂	0.01 <i>M</i> <i>l</i> (+)-Glutamine	0.28	0.30
		0.0033 <i>M</i> NH ₄ Cl	0.26	0.30
		0.0033 <i>M</i> NH ₄ Cl; 0.02 <i>M</i> pyruvate	0.31	0.30
	5% CO ₂ in O ₂	0.01 <i>M</i> Glutamine	1.08	0.89
		0.0033 <i>M</i> NH ₄ Cl	0.64	0.89
		0.0033 <i>M</i> NH ₄ Cl; 0.02 <i>M</i> pyruvate	1.20	0.89
5	"	0.01 <i>M</i> <i>l</i> (+)-Glutamine	1.01	0.36
		0.02 <i>M</i> <i>l</i> (+)-Glutamine	1.12	0.36
		0.04 <i>M</i> <i>l</i> (+)-Glutamine	1.21	0.36
		0.005 <i>M</i> NH ₄ Cl	0.70	0.36
		0.01 <i>M</i> NH ₄ Cl	0.53	0.36
		0.02 <i>M</i> NH ₄ Cl	0.44	0.36
6	"	0.01 <i>M</i> NH ₄ Cl	0.79	1.07
		0.01 <i>M</i> NH ₄ Cl; 0.02 <i>M</i> pyruvate	2.12	1.07
		0.01 <i>M</i> NH ₄ Cl; 0.02 <i>M</i> oxaloacetate	2.34	1.07
7	"	0.01 <i>M</i> NH ₄ Cl; 0.02 <i>M</i> oxaloacetate	1.83	0.72
		0.01 <i>M</i> NH ₄ Cl; 0.02 <i>M</i> oxaloacetate; 0.01 <i>M</i> <i>l</i> (+)-alanine	1.72	0.72
		0.01 <i>M</i> NH ₄ Cl; 0.02 <i>M</i> oxaloacetate; 0.01 <i>M</i> <i>l</i> (+)-ornithine	2.11	0.72
		" 0.01 <i>M</i> <i>l</i> (+)-arginine	1.94	0.72
		" 0.01 <i>M</i> <i>dl</i> -serine	1.49	0.72
		" 0.01 <i>M</i> <i>l</i> (-)-asparagine	1.85	0.72
		" 0.01 <i>M</i> <i>l</i> (-)-aspartate	1.61	0.72
Phosphate saline				
8	O ₂	0.01 <i>M</i> NH ₄ Cl	0.67	0.63
		0.01 <i>M</i> NH ₄ Cl; 0.02 <i>M</i> pyruvate	0.92	0.63
		0.01 <i>M</i> NH ₄ Cl; 0.02 <i>M</i> oxaloacetate	2.45	0.63
NaHCO ₃ saline				
9	5% CO ₂ in O ₂	0.01 <i>M</i> <i>l</i> (+)-Ornithine	1.36	0.45
		0.01 <i>M</i> <i>l</i> (-)-Leucine	1.20	0.45
		0.01 <i>M</i> <i>l</i> (+)-Valine	0.99	0.45
		0.02 <i>M</i> <i>dl</i> -Lysine	0.80	0.45
		0.01 <i>M</i> <i>l</i> (-)-Methionine	0.94	0.45
		0.01 <i>M</i> <i>l</i> (-)-Histidine	1.01	0.45
10	"	0.01 <i>M</i> NH ₄ Cl; 0.02 <i>M</i> oxaloacetate	1.41	0.52
		" 0.02 <i>M</i> glucose	0.78	0.52
		" 0.5% glycogen	1.05	0.52
		" 0.02 <i>M</i> citrate	1.26	0.52
		" 0.02 <i>M</i> α-glycerophosphate	1.26	0.52
		" 0.02 <i>M</i> <i>l</i> (+)-lactate	0.56	0.52
		" 0.02 <i>M</i> pyruvate	0.82	0.52
11	"	"	1.55	0.81
		" 0.02 <i>M</i> citrate	1.44	0.81
		" 0.02 <i>M</i> oxaloacetate	2.62	0.81
		" 0.02 <i>M</i> pyruvate	1.92	0.81
		0.02 <i>M</i> <i>l</i> (+)-Glutamine	1.67	0.81
		0.02 <i>M</i> <i>l</i> (+)-Glutamine; 0.01 <i>M</i> NH ₄ Cl; 0.02 <i>M</i> oxaloacetate	3.40	0.81
		0.02 <i>M</i> <i>l</i> (+)-Citrulline	0.92	0.81
		0.02 <i>M</i> <i>l</i> (+)-Citrulline; 0.01 <i>M</i> NH ₄ Cl; 0.02 <i>M</i> oxaloacetate	2.38	0.81

(4) Of the non-nitrogenous substances (when added together with NH_4Cl) oxaloacetate effects the greatest increase of the rate of hypoxanthine synthesis (Exps. 1, 3, 6, 8, 10).

(5) When glutamine, NH_4Cl and oxaloacetate are added together a summation of the effects sometimes occurs (Exp. 11).

(6) Amino-acids, when added together with NH_4Cl and oxaloacetate, have no significant effect on the hypoxanthine synthesis (Exps. 7, 11). There is thus no effect comparable with the action of ornithine on the urea synthesis in rat liver. This negative result does exclude the possibility that the substances tested play a part in the hypoxanthine synthesis, as a positive effect can only be expected if the concentration of the substance tested is a "limiting factor".

Hypoxanthine formation in other tissues

The livers of the fowl, duck, rat and guinea-pig were treated in the same way as pigeon liver, but no appreciable quantities of hypoxanthine were found when the conditions were aerobic. This may be expected since added hypoxanthine disappears in these tissues; they all contain xanthine oxidase. Anaerobically small quantities of hypoxanthine were liberated in these tissues, $Q_{\text{hypoxanthine}}$ being of the order 0.2.

Effects of glutamine and oxaloacetate on the uric acid synthesis in fowl liver

In view of the effects of oxaloacetate and glutamine on the hypoxanthine synthesis in pigeon liver we thought it of interest to investigate the effects of these substances on the corresponding reaction in fowl liver, the uric acid synthesis. Uric acid was determined colorimetrically, as described in the preceding paper. $Q_{\text{uric acid}}$, the rate of uric acid synthesis, is expressed in terms equivalent to $Q_{\text{hypoxanthine}}$. The results, recorded in Table III show that the corresponding effects do in fact occur.

Table III. *Effect of glutamine and oxaloacetate on the uric acid formation in fowl liver*

Substrates added (final concentration)	$Q_{\text{uric acid}}$
—	0.41
0.02 <i>M</i> Oxaloacetate; 0.01 <i>M</i> NH_4Cl	1.32
0.02 <i>M</i> $\text{L}(+)$ Glutamine	1.33
0.02 <i>M</i> Pyruvate; 0.01 <i>M</i> NH_4Cl	1.04

DISCUSSION

Reindel & Schuler's results. Although no explanation can be offered for the findings of Reindel & Schuler [1937] mentioned in the introduction, it appears certain from our experiments that xanthine is not formed *synthetically* in pigeon liver. It may be possible that traces of xanthine arise *hydrolytically* from guanine, or guanine nucleosides or nucleotides, since pigeon liver contains a guanase; but the quantities of guanine compounds present in the liver are almost negligible compared with the amounts of hypoxanthine synthesized in the presence of an excess of NH_4 salts.

The effect of oxaloacetate. The stimulating effect of oxaloacetate suggests the idea that oxaloacetate serves as the source of the carbon skeleton in the synthesis of the purine ring. Oxaloacetate, as an intermediate product in the oxidative

breakdown of carbohydrate, lactate and pyruvate belongs to the relatively small number of substances available in sufficient quantities.

The effect of glutamine. The facts at our disposal are not sufficient to explain the action of glutamine. It is improbable that the carbon chain of the glutamine molecule, or part of it, enters directly into the purine ring, but more likely that glutamine acts as an "ammonia carrier". It is of great interest in this connexion that Leuthardt [1938] found a somewhat analogous effect of glutamine on the urea synthesis in the liver of starved guinea-pigs.

These effects of glutamine, however, are not necessarily to be explained by a direct participation in the synthesis, since synthetic reactions are always complex processes connected with the production and transmission of energy. Several observations, such as the occurrence of a glutamine synthesis in brain cortex and in retina [Krebs, 1935] and the necessity of glutamine for the growth of *Streptococcus haemolyticus* [McIlwain *et al.* 1939], suggest that glutamine plays a more general, though still entirely undefined, role in cell metabolism.

SUMMARY

1. The purine base synthesized by pigeon liver requires 1 mol. O_2 for its quantitative conversion into uric acid. The base is therefore hypoxanthine. Contrary results of Reindel & Schuler [1937] who claim to have found a formation of xanthine remain unexplained.

2. The effects of added substrates on the rate of hypoxanthine synthesis were studied. Glutamine and oxaloacetate were found to increase the rate of the synthesis. The cause of these effects is discussed.

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