CXCVI. THE SYNTHESIS OF URIC ACID IN THE AVIAN ORGANISM: HYPOXANTHINE AS AN INTERMEDIARY METABOLITE.

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It has been demonstrated that the co-operation of two organs, liver and kidney, is necessary for uric acid synthesis in pigeons [Krebs & Benzinger, 1933; Schuler & Reindel, 1933, 1, 2]. On this account it is possible to resolve the complex process into two parts which may be studied individually. In this paper it will be shown that the primary process is a ring synthesis, occurring in the liver and yielding hypoxanthine, which is then oxidised to uric acid by the kidney (or by certain other tissues) under the influence of xanthine oxidase. Thus in pigeons xanthine oxidase is a component of the system which synthesizes uric acid.

METHODS.

The tissue slice technique was employed, the medium being the physiological saline originally devised for mammalian tissues [Krebs & Henseleit, 1932]. This procedure is permissible, because the ionic composition of bird's serum does not differ significantly from that of mammalian serum [Dyer & Roe, 1934; Morgan & Chichester, 1935]. The concentration of bicarbonate was 0.025 *M* and the saline was in equilibrium with an atmosphere of 5% CO₂ + 95% O₂. The *pH* was 7.4. Substrates were added in neutral solution: acids as sodium salts, ammonia as chloride.

The slices were prepared in the usual way and washed with saline as described by Krebs & Henseleit [1932]. The temperature of the thermostat was 40° unless otherwise stated. The tissue (10–20 mg. dry weight) was immersed in 3.0 ml. saline and shaken usually for 2 hours, after which the change in concentration of metabolites was sufficient for accurate determination.

Uric acid was estimated manometrically [Edson & Krebs, 1936].

Quantities of metabolites are expressed as μ l. gas in the customary way. 17 mg. ammonia are considered equivalent to 22,400 μ l. and 1 millimol uric acid (168 mg.) is made equivalent to $2 \times 22,400$ μ l. CO₂, since 1 molecule uric acid yields 2 molecules CO₂ in the determination.

The rate of metabolism is expressed by the following quotients:

$$Q_{\text{NH}_3} = \frac{\mu\text{l. NH}_3}{\text{mg. tissue (dry weight)} \times \text{hours}},$$
$$Q_{\text{uric acid}} = \frac{\mu\text{l. CO}_2}{\text{mg. tissue (dry weight)} \times \text{hours}}.$$

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The site of uric acid formation in pigeons.

It is known that pigeon liver and kidney slices form mere traces of uric acid when the tissues are examined separately, but considerable quantities are produced if both tissues are present together in ammonia-containing saline [Krebs & Benzinger, 1933; Schuler & Reindel, 1933, 1]. Schuler & Reindel [1935] published experiments, moreover, which suggest that pancreas slices may replace kidney. Experiments in which uric acid was determined manometrically are in agreement with these previous observations (Table I).

Table I. *Uric acid production by pigeon tissues.*

Tissue present (mg.)	Uric acid formed in 2 hrs. μ l. CO ₂
1. Liver (21.03)	9.1
2. Kidney (11.59)	10.6
3. Pancreas (14.99)	13.4
4. Liver (13.67) + kidney (15.89)	56.6
5. Liver (19.24) + pancreas (5.92)	35.0

The disappearance of ammonia.

In attempting to elucidate the separate functions of the relevant tissues we first identified the tissue that binds ammonia. Slices of the different tissues were shaken alone in saline containing ammonium chloride, and after 1 or 2 hours

Table II. *Disappearance of ammonia in the presence of bird's liver slices.*

Liver mg.	State of nutrition	Substrate	Amount of NH ₃ per flask μl.		Duration of exp.	NH ₃ used μl.	-Q _{NH₃}
			Initial	Final			
I. Pigeon.							
15-66	Starved	Nil	406	314	2 hrs.	92	2.93
13-47	48 hrs.	<i>dl</i> -Lactate, 0.02 <i>M</i>	„	157		249	9.24
13-48		Pyruvate, 0.02 <i>M</i>	„	104		302	11.2
13-01		Glycerol, 0.02 <i>M</i>	„	294		112	4.30
13-37		α-Ketoglutarate, 0.02 <i>M</i>	„	270		136	5.09
II. Pigeon.							
28-94	Starved	Nil	390	135	2 hrs.	255	4.41
22-75	24 hrs.	Succinate, 0.02 <i>M</i>	„	175		215	4.72
24-38		α-Glycerophosphate, 0.02 <i>M</i>	„	262		128	2.63
19-49		Pyruvate, 0.02 <i>M</i>	„	26		364	9.34
24-13		Acetate, 0.02 <i>M</i>	„	295		95	1.97
20-53		<i>l</i> (+)-Glutamate, 0.02 <i>M</i>	„	207		183	4.46
25-90		Glucose, 0.02 <i>M</i>	„	149		241	4.65
28-50		Glycine, 0.02 <i>M</i>	„	168		222	3.90
23-92		Glycerol, 0.02 <i>M</i>	„	81		309	6.46
22-72		<i>n</i> -Octanoate, 0.02 <i>M</i>	„	293		97	2.13
29-98	Ornithine, 0.02 <i>M</i>	„	142		248	4.14	
III. Pigeon.							
8-69	Well fed	Nil	448	240	2 hrs.	208	12.0
7-16		<i>dl</i> -Lactate, 0.018 <i>M</i>	„	213		235	16.5
IV. Pigeon.							
19-23	Well fed	Nil	515	278	100 min.	237	7.40
14-95		Pyruvate, 0.01 <i>M</i>	„	156		359	14.2
V. Fowl.							
16-61	Well fed	Nil	416	182	2 hrs.	234	7.04
21-29		<i>dl</i> -Lactate, 0.01 <i>M</i>	„	140		276	6.49
20-50		Pyruvate, 0.01 <i>M</i>	„	57		359	8.75
19-90		β-Phosphoglycerate, 0.01 <i>M</i>	„	172		244	6.14
21-19		α-Ketoglutarate, 0.01 <i>M</i>	„	105		311	7.07
19-73		α-Glycerophosphate, 0.01 <i>M</i>	„	110		306	7.75

ammonia was determined by the method of Parnas. Neither kidney nor pancreas removed added ammonia, whereas liver caused the disappearance of large amounts. Table II shows the rate of ammonia disappearance in pigeon's and fowl's liver in the presence of various substrates. We added these substances expecting to obtain information about the source of the carbon skeleton of uric acid. Although lactic and pyruvic acids have a more marked effect than other substrates especially in the starved animal, it seems premature in view of the complex nature of the systems involved to draw definite conclusions from this fact.

The effect of xanthine oxidase prepared from milk.

The traces of uric acid formed by pigeon's liver account for about 5% of the ammonia which has disappeared. If, however, the total nitrogen in solution is determined by Kjeldahl's procedure, no significant decrease is observed during ammonia disappearance. This shows that the nitrogenous substance which is formed from ammonia, and which is not uric acid, is returned to the solution by the tissue slices, and the problem of its identity arises.

It is characteristic of this substance that it yields uric acid with kidney and pancreas of the pigeon. The work of Morgan [1926] suggested to us that the substance in question might be xanthine or hypoxanthine, because Morgan gave the following distribution of the enzyme in birds:

		Xanthine oxidase
Pigeon	Liver	-
	Pancreas	+
	Kidney	+
Fowl	Liver	+

We have confirmed these observations, using a more sensitive technique recently developed by Booth [1935].

The findings of Morgan are therefore in agreement with the view that the action of xanthine oxidase is the final step in uric acid synthesis in birds, which requires a second tissue in the pigeon but not in the fowl. In order to test this assumption we attempted to replace kidney and pancreas by a xanthine oxidase preparation ("wey preparation") made from milk according to the directions of Dixon & Kodama [1926]. The results in Table III show that the assumption is correct: pigeon's liver produces a substance which gives uric acid in presence of xanthine oxidase.

Table III. *Effect of milk xanthine oxidase on the pigeon's uric acid precursor.*

Tissue and enzyme	Uric acid formed in 2 hrs. μ l. CO ₂
1. Liver (21.03 mg.)	9.1
2. Xanthine oxidase (50 mg.)	3
3. Liver (23.02 mg.) + xanthine oxidase (50 mg.)	63

In view of the high specificity of xanthine oxidase it is probable that the uric acid precursor is either xanthine or hypoxanthine. The following measurement of the relation between oxygen uptake and uric acid formation shows that the substance behaves like hypoxanthine.

Pigeon liver slices (303 mg.) were shaken for 2 hours in ammonia-containing saline (50 ml.), and after removal of the slices bicarbonate was decomposed with hydrochloric acid, and phosphate buffer (pH 7.4) was added. 3.0 ml. of this

solution were measured into a manometric cup and 0.1 ml. xanthine oxidase solution was placed in the side-bulb. The flask was equilibrated in the thermostat and the oxygen uptake was measured after addition of xanthine oxidase. When the oxygen uptake ceased uric acid was determined (Table IV).

Table IV. *Oxygen uptake and uric acid formation under the influence of xanthine oxidase.*

3.0 ml. Ringer solution in which pigeon liver slices had been shaken for 2 hrs. + xanthine oxidase.

Time min.	Oxygen uptake $\mu\text{l. O}_2$	Uric acid $\mu\text{l. CO}_2$
10	36.1	—
20	44.2	—
30	45.5	—
40	45.5	87.0

(1 *mM* uric acid = 2 *mM* CO_2)

If xanthine were the only substrate undergoing oxidation to uric acid, the molecular ratio O_2 consumed/uric acid formed would have the value 0.5, or if it were hypoxanthine, 1.0. The observed value is $45.5/43.5 = 1.04$ which suggests that the purine base is hypoxanthine rather than xanthine. It should be mentioned that added xanthine and hypoxanthine yield the theoretical ratios under the conditions of the experiment recorded in Table IV.

The presence of the purine base is easily demonstrated by the methylene blue technique. Ringer solution containing ammonium salt in which pigeon liver slices have been shaken will reduce methylene blue rapidly if xanthine oxidase be added. 5.0 ml. of the solution mentioned above (303 mg. liver in 50 ml. saline) were mixed in an evacuated Thunberg tube with 0.2 ml. methylene blue solution (0.1 %) and 0.3 ml. 20 % xanthine oxidase suspension at *pH* 8.0. Reduction was complete in $3\frac{1}{2}$ min. at 40° . A control without xanthine oxidase showed no reduction in 24 hours.

The isolation of hypoxanthine.

The above conclusions have been confirmed by the isolation and identification of hypoxanthine. The liver of a pigeon was cut into slices which were evenly distributed amongst three large shaking vessels, each of which contained 40 ml. of bicarbonate-Ringer solution. The Ringer solution was 0.02 *M* with respect to pyruvate and 0.007 *M* with respect to ammonium chloride. The slices were shaken for 2 hr. at 40° , the gas space containing a mixture of 5 % CO_2 and 95 % O_2 .

After removal of the slices the fluid was deproteinized with 0.1 volume of 30 % trichloroacetic acid, and the purine base was separated by the method of Salkowski [1898]. The protein-free filtrate was treated with 20 ml. strong ammonia in order to remove phosphates. Since the calcium and magnesium ions present were more than equivalent to the phosphate ions the use of magnesia mixture was unnecessary. The clear filtrate (after phosphate precipitation) was reduced to one-eighth its original volume by evaporation on the water-bath. Purine-containing extracts from the livers of six pigeons were treated in the same way and combined, made strongly alkaline with ammonia and the purine precipitated with 10 ml. 5 % silver nitrate solution. The yellowish, gelatinous flocculum of silver purine was separated by centrifuging, washed twice with dilute ammonia and finally with distilled water. Previous tests had shown the absence of uric acid from the extract, and therefore none of the usual precautions for the separation of that substance was necessary.

The purine silver compound was dissolved in hot nitric acid (sp. gr. 1.1), from which it crystallized in small white needles immediately on cooling. This behaviour is characteristic of hypoxanthine silver nitrate. The product was recrystallized three times from hot nitric acid of sp. gr. 1.1. From 3.86 g. of liver (dry weight) 43 mg. hypoxanthine silver nitrate were obtained. In other experiments the yields were from 4.51 g. liver 55 mg. and from 5.60 g. liver 74 mg. hypoxanthine silver nitrate.

Free hypoxanthine was prepared by decomposing the silver nitrate compound with hydrogen sulphide. 30 mg. hypoxanthine silver nitrate were suspended in 20 ml. distilled water and the liquid saturated with H_2S . The filtrate from the silver sulphide was warmed and aerated to remove H_2S ; it was then made weakly alkaline with ammonia and aerated again until it was free from ammonia and was then evaporated on the water-bath until the volume was 2 ml. When the solution was cooled in the ice-box, a finely divided, white substance was deposited; yield, 10 mg. This was recrystallized three times from distilled water and dried *in vacuo* for several days before analysis.

Elementary analysis (Dr Weiler, Oxford)

	C	H	N (Dumas)
Substance isolated	44.70	3.25	39.86
Substance isolated	—	—	39.48
Calculated for hypoxanthine	44.10	2.96	41.17
Calculated for xanthine	39.46	2.65	36.84
Pure hypoxanthine (Hoffmann-La Roche)	44.87	3.43	40.31
Pure hypoxanthine (Hoffmann-La Roche)	—	—	39.11

The values for the nitrogen (Dumas) tend to be low, but pure hypoxanthine gives the same low figures and the agreement between the figures obtained for hypoxanthine and for the substances isolated from liver is very satisfactory.

Properties of the substance isolated.

1. A white, micro-crystalline, non-deliquescent powder, sparingly soluble in cold water but rather more soluble in hot water.

2. The solution reduced methylene blue anaerobically in presence of xanthine oxidase.

3. When a solution of the substance was shaken with xanthine oxidase in air, it absorbed oxygen and formed uric acid.

4. It formed a salt with nitric acid and silver nitrate. When this salt was treated according to Strecker [1858], it gave a compound with the properties of hypoxanthine silver oxide; N, 15.96%, the theoretical value being 15.63% [Bruhns, 1890].

The above facts establish the identity of the uric acid precursor as hypoxanthine. This is the purine which Schuler and Reindel [1935] have recently stated to be formed by pigeon's liver.

The influence of cell structure.

There is no disappearance of ammonia in preparations of liver, the cell structure of which has been destroyed by processes such as grinding with sand.

It has been mentioned that kidney slices can be replaced effectively by xanthine oxidase prepared from milk. Further, a kidney preparation made by the method of Booth [1935] is an adequate substitute for slices, although cell structure is destroyed.

The quantitative relationship between ammonia disappearance and hypoxanthine formation.

Determinations of hypoxanthine show that the amount of hypoxanthine formed accounts for not more than 30% of the ammonia disappearing in the presence of lactate and pyruvate. There are other ammonia-binding mechanisms in liver and large quantities of amino-nitrogen are formed under some conditions, especially in the presence of lactate or pyruvate. These data and the methods employed will be published in a later communication.

Uric acid synthesis in other birds.

The presence of hypoxanthine could not be detected in saline in which fowl's or duck's liver slices had been shaken, but this does not contradict the hypothesis that hypoxanthine may also be the direct precursor of uric acid in these birds as well as in the pigeon, since the xanthine oxidase activity of hen's and duck's liver is so high that hypoxanthine would be removed more quickly than it was formed by synthesis. The experiments of Table V indicate that this is the case.

Table V. *Rates of uric acid formation from ammonium chloride and from hypoxanthine in fowl and duck livers.*

Animal	Substrate	$Q_{\text{uric acid}}$
1. Fowl	NH ₄ Cl; 0.01 <i>M</i> pyruvate	1.15
	Hypoxanthine 0.0025 <i>M</i>	1.97
2. Duck	NH ₄ Cl; 0.01 <i>M dl</i> -lactate	0.96
	Hypoxanthine 0.0025 <i>M</i>	4.76

SUMMARY.

1. In pigeons two tissues are necessary for uric acid synthesis. The primary step, the binding of ammonia with some uncertain source of carbon, occurs in the liver where hypoxanthine is formed.
2. The final conversion into uric acid, which takes place in the kidney (or pancreas), is an oxidation catalysed by xanthine oxidase.
3. There is evidence suggesting that hypoxanthine is also an intermediate in other birds.

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