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The Metabolism of 3-Hydroxyanthranilic and Quinolinic Acids in the Chick Embryo

By E. QUAGLIARIELLO AND G. DELLA PIETRA

Institute of Chemistry, Faculty of Medicine and Surgery, University of Naples

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3-Hydroxyanthranilic acid (I) occupies a central position in the biological degradation of tryptophan (for discussion, see Cedrangolo, 1953). It can replace nicotinic acid in the growth of the rat (Wiss, Viollier & Müller, 1949); furthermore, it is readily converted *in vitro* into quinolinic acid (II) (Priest, Bokman & Schweigert, 1951).

Makino, Itoh & Nishi (1951) claimed to have demonstrated *in vitro* the conversion by mammalian liver of 3-hydroxyanthranilic and quinolinic acids into nicotinic acid, but Cedrangolo & Scardi (1951) raised the objection that the long incubation time in these experiments might have induced bacterial growth and therefore yielded unreliable results. In fact, Henderson, Hill, Koski & Weinstein (1951) and Hellmann & Wiss (1952) failed to confirm the results of Makino *et al.* (1951); and Denton, Kellogg, Rowland & Bird (1952) and Cedrangolo, Quagliariello & Della Pietra (1953), after injecting 3-hydroxyanthranilic acid into hen's eggs, were unable to show any formation of nicotinic acid during incubation.

Quinolinic acid also promotes rat growth (Henderson, 1949) and appears in the urine after administration of tryptophan (Schayer & Henderson, 1952), but in experiments *in vitro* Cedrangolo & Jannella (1950) and Cedrangolo & Scardi (1951) were unable to find any conversion of quinolinic into nicotinic acid in homogenates and tissue slices from liver, kidney, spleen, brain and muscle of rats

and rabbits. Additional support for the absence of conversion of quinolinic into nicotinic acid was given by Cedrangolo, Della Pietra & Quagliariello (1953), who found that quinolinic acid, injected into hen's eggs, did not cause any increase in the content of nicotinic acid during incubation.

Given this conflicting evidence, it was thought worth while to examine whether 3-hydroxyanthranilic acid, and possibly quinolinic acid as well, could be converted into nicotinic acid by chick embryo. Short preliminary communications of the results referred to in this paper have already appeared elsewhere (Quagliariello & Della Pietra, 1954; Della Pietra, Quagliariello & Auricchio, 1954).

EXPERIMENTAL AND RESULTS

We first investigated whether 3-hydroxyanthranilic acid could be converted into nicotinic acid.

Chick embryos on the 12th incubation day were homogenized in a Potter glass homogenizer with Krebs-Ringer-phosphate solution (5 ml./g. tissue). The incubation mixture consisted of 5 ml. of homogenate, 3 mg. of 3-hydroxyanthranilic acid (Hoffman-La Roche and Co.), and Krebs-Ringer-phosphate, pH 7.3; total vol. 15 ml. The incubation took place at 37° for 3 hr., with oxygen as the gas phase. In order to autolyse the tissues, the incubation was then continued under benzene for 21 hr. The volume was then brought to 40 ml. with water, and, after autoclaving for 20 min. at 121°, the volume was readjusted to 40 ml. and the suspension filtered. Samples of the filtrate were taken for the quantitative analysis of nicotinic acid by the microbiological technique of Della Pietra & Quagliariello (1953), using *Lactobacillus arabinosus*. Blanks with tissue only (A), with 3-hydroxyanthranilic acid only (B), and with tissue + 3-hydroxyanthranilic acid added at the end of incubation (C), were run in parallel through the entire procedure. Values of A and C were always identical; values of B were nil.

Table 1 shows the results of our experiments. Some extra nicotinic acid was formed when homogenates of embryonic tissue were incubated with 3-hydroxyanthranilic acid.

We next investigated the conversion of quinolinic acid into nicotinic acid.

Chick embryos on the 12th day of incubation were used. The experimental technique was the same as before. Quinolinic acid was obtained from Bios Laboratories Inc. In some tests, the autolysis under benzene was omitted and autoclaving was carried out immediately after the incubation, as

autoclaving in a neutral medium does not decarboxylate quinolinic acid.

Blanks with tissue alone, with quinolinic acid alone, and with tissue + quinolinic acid added before autoclaving, were also included.

Table 2 shows the results of the experiments, and indicates that quinolinic acid, under the experimental conditions employed, is not converted into nicotinic acid.

The position of quinolinic acid was not clarified by the experiments described above, as this compound, which is a normal constituent of hen's eggs (Quagliariello & Della Pietra, 1953), could be either an end product of a secondary route from 3-hydroxyanthranilic acid, or have no relation to tryptophan metabolism. Investigations were therefore carried out to find whether extracts from chick embryo could perform a partial transformation *in vitro* of 3-hydroxyanthranilic into quinolinic acid.

The chick-embryo homogenate was diluted with 4 vol. of Krebs-Ringer-phosphate, and centrifuged. The sediment was discarded.

The supernatant was incubated with 3-hydroxyanthranilic acid at 37° for 150 min. in air. Quinolinic acid was determined by the method of Rabinovitz, Fineberg & Greenberg (1953), with minor modifications. The amount of quinolinic acid formed was calculated by subtracting the value for the embryonic extract alone, and also the value with 3-hydroxyanthranilic acid alone (assay with 3-hydroxyanthranilic acid minus blank with the reagents alone), from the value for the complete system. In these experiments an extract from chick embryo (12th incubation day) was used,

Table 1. *Formation of nicotinic acid from 3-hydroxyanthranilic acid by 12-day chick-embryo homogenates*

Expt.	Nicotinic acid (μ g./g. embryo)	
	Embryo incubated alone	Embryo incubated with 3-hydroxyanthranilic acid
1	32.4	41.6
2	31.3	40.8
3	32.1	36.6
4	26.8	45.2
5	26.7	34.0
6	28.0	36.0
7	24.8	39.2
8	17.0	32.0
9	26.1	31.0
10	27.3	32.4
11	40.0	42.8
12	26.4	38.4
13	27.6	33.6
14	36.2	42.4
15	21.2	27.2
16	20.6	23.7
Average	27.7	36.2
	(s.d. 5.5)	(s.d. 5.7)

Table 2. *Content of nicotinic acid in 12-day chick-embryo homogenates incubated with quinolinic acid*

Expt.	Quinolinic acid (mg.)	Incubation time (hr.)	Nicotinic acid (μ g./g. embryo)	
			Embryo incubated alone	Embryo incubated with quinolinic acid
1	3	5	26.5	28.0
2	3	5	44.0	36.0
3	3	5	29.2	29.2
4	3	3	33.6	28.0
5	3	3	25.2	23.2
6	3	3	20.4	22.8
7	2	3	24.8	22.4
8	2	3	22.0	25.6
9	2	3	22.4	23.2
10	2	3	22.4	20.8
11	2	3	22.4	20.4
12	2	3	22.8	21.6
13	2	3	21.6	22.8
14	1	3	23.7	20.0
15	1	3	26.4	24.0
16	1	3	31.2	22.8
17	0.5	3	22.8	21.2
18	0.5	3	20.0	25.2
Average			25.6	23.2

because in similar experimental conditions Rabinovitz *et al.* (1953), with rat liver, observed a marked conversion of 3-hydroxyanthranilic into quinolinic acid.

Table 3 gives the experimental results. It can be seen that no conversion of 3-hydroxyanthranilic into quinolinic acid was demonstrable under these experimental conditions.

Table 3. *Content of quinolinic acid after incubation of the supernatant of chick-embryo homogenate with 3-hydroxyanthranilic acid*

3-Hydroxy-anthranilic acid (μ moles)	Quinolinic acid added*	Quinolinic acid found (μ moles)
19.6	20	20.06
19.6	10	9.93
9.8	20	19.74
9.8	10	10.00
6.5	20	19.87
6.5	10	9.35

* Added after incubation and deproteinization.

Table 4. *Recovery of 3-hydroxyanthranilic acid after incubation with chick-embryo homogenates*

Time of incubation (min.)	3-Hydroxy-anthranilic acid added (μ g.)	3-Hydroxy-anthranilic acid recovered (μ g.)
180	3000	3000
30	600	608
30	500	510
30	400	400
30	300	302
30	200	198
30	150	144
15	500	500
15	150	147

Since the conversion of 3-hydroxyanthranilic into nicotinic acid in our experiments amounted to only 0.3 %, it was possible that 3-hydroxyanthranilic acid was converted into compounds other than nicotinic acid. We therefore followed the fate of 3-hydroxyanthranilic acid under experimental conditions in which formation of nicotinic acid had been observed. Whole homogenates of 12-day chick embryos were used, and 3-hydroxyanthranilic acid was determined according to Bokman & Schweigert (1950) with minor modifications.

Each set of experiments required its own standard curve, because alterations in the incubation time gave widely different readings for equal amounts of 3-hydroxyanthranilic acid. The sensitivity of this method does not permit determination of the disappearance of the small amount of 3-hydroxyanthranilic acid which is converted into nicotinic acid, whereas the microbiological method was able to demonstrate the formation of such small amounts of nicotinic acid.

The results presented in Table 4 show that practically the whole of the 3-hydroxyanthranilic acid was recovered at the end of the incubation time. Each value is the mean of many similar experiments. Times of incubation shorter than in the experiments reported in Table 1 were sometimes used because Bokman & Schweigert (1950) had demonstrated the total disappearance in 5 min. of 3-hydroxyanthranilic acid incubated with rat liver.

DISCUSSION

Snell & Quarles (1941) have shown that on the 12th day of incubation a large synthesis of nicotinic acid is observed in hen's eggs. It has also been shown that tryptophan is the source of nicotinic acid synthesized in developing hen's eggs (Quagliariello, Fidanza, Della Pietra & Scala, 1952). On these grounds 12-day chick embryos were chosen for our *in vitro* experiments on the metabolism of 3-hydroxyanthranilic and quinolinic acids. Our results suggest the conversion of 3-hydroxyanthranilic into nicotinic acid to a small but definite extent, whereas a conversion of quinolinic into nicotinic acid was not demonstrated. It seems probable that no metabolite other than nicotinic acid originated from 3-hydroxyanthranilic acid.

The formation of quinolinic acid from 3-hydroxyanthranilic acid could not be demonstrated. Our experiments suggest that nicotinic acid in chick embryo is formed from 3-hydroxyanthranilic acid either directly or by way of a still unknown intermediate, and that quinolinic acid does not take part directly or indirectly in the metabolic processes leading from tryptophan to nicotinic acid. The conversion of 3-hydroxyanthranilic acid into nicotinic acid in our experiments is of the order of only 0.3 %, but this value is comparable with that obtained by Schweigert, German & Garber (1948) for the conversion of tryptophan into nicotinic acid.

Some results still remain to be explained, such as the presence of quinolinic acid in hen's eggs (Quagliariello & Della Pietra, 1953) and its increase from the 1st to the 18th day of incubation. The reason for the absence of formation of extra nicotinic acid after injection of 3-hydroxyanthranilic acid into the incubating eggs (Denton *et al.* 1952; Cedrangolo, Quagliariello & Della Pietra, 1953) is also not clear.

SUMMARY

Homogenates from 12-day-old chick embryos convert 3-hydroxyanthranilic acid into nicotinic acid to a small extent but apparently not into quinolinic acid or into other compounds. Quinolinic acid is not converted into nicotinic acid.

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Cephalosporin P₁ and Helvolic Acid

By H. S. BURTON,*† E. P. ABRAHAM* AND H. M. E. CARDWELL†

Dyson Perrins Laboratory and Sir William Dunn School of Pathology, University of Oxford

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The isolation of antibiotics from the culture fluid of a species of *Cephalosporium* was described by Burton & Abraham (1951). These substances, which were called cephalosporin P₁, P₂, P₃, P₄ and P₅, appeared to belong to the same chemical family. They resembled helvolic acid, an antibiotic produced by *Aspergillus fumigatus* mut. *helvola* Yuill (Chain, Florey, Jennings & Williams, 1943) in some of their chemical and biological properties. All the antibiotics were inactivated by an enzyme produced by certain strains of aerobic spore-forming organisms. A strain of *Staphylococcus aureus* which had become resistant to cephalosporin P₁ was found to be resistant to helvolic acid and vice versa.

Of the members of the cephalosporin P family, only cephalosporin P₁ was obtained in a quantity sufficient for detailed chemical study. This paper records some observations on the nature of cephalosporin P₁ (subsequently referred to as cephalosporin), and its relationship to helvolic acid. Cephalosporin (m.p. 147°; $[\alpha]_D^{20} + 28^\circ$) is an acid which contains carbon, hydrogen and oxygen only. The results of elementary analysis were difficult to

interpret until it was realized that the antibiotic crystallized from hydroxylic solvents with solvent of crystallization, but the empirical formula C₃₂H₄₈O₈ now appears to be firmly established. The molecular weight of material crystallized from aqueous ethanol (dried *in vacuo* over P₂O₅), determined by X-ray diffraction measurements by Miss M. Curzon and Dr Dorothy Hodgkin, was 601 ± 16. This value, and the analytical figures, are in excellent agreement with the molecular formula C₃₂H₄₈O₈·H₂O, $\frac{1}{2}$ C₂H₅OH.

Cephalosporin, like helvolic acid, is a monobasic carboxylic acid. Electrometric titration in 60% (v/v) ethanol showed the presence of a group with a pK of 6.8 (equivalent weight 610). This would correspond to a pK of approximately 4.9 in water, i.e. to that expected of an aliphatic carboxyl group not linked closely to a strongly electronegative centre. The infrared spectrum of cephalosporin showed a broad band at 5.77–5.86 μ. due to the presence of carbonyl groups. In the potassium salt a new band, assigned to the –CO₂–group, appeared at 6.45 μ., but a broad band at 5.8 μ. still remained.

On methylation with diazomethane at 0° cephalosporin forms a non-acidic monomethyl ester, C₃₃H₅₀O₈ (m.p. 196°; $[\alpha]_D^{10} + 28^\circ$). This ester, like methyl helvolate, melts some 50° higher than

* Sir William Dunn School of Pathology, University of Oxford.

† Dyson Perrins Laboratory, University of Oxford.