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SOME DATA ON THE IN VITRO HYDROXYLATION OF ANTHRANILIC ACID AND ITS BIOLOGICAL TRANSFORMATION PRODUCTS (PART 17)

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Introduction

The hydroxylation of the group of compounds mentioned in the summary has been dealt with earlier (MATKOVICS—GÖNDÖS, 1968; MATKOVICS—MARIÁN, 1969a; MATKOVICS—MARIÁN, 1966b). The aim of this paper is to review such 1969a; MATKOVICS—MARIÁN, 1969b). The aim of this paper is to review such results.

Anthranilic acid (I), indole (VI) and its derivatives (VII—IX) form a very important biological and pharmacological group of compounds and are related metabolically. This group plays an important role not only in the animal organism but in plants too.

3-Hydroxynathranilic acid has long been known as one of the products of the in vivo tryptophane metabolism in the animal organism (among others KARLSON (1970). However, little is known about the in vivo and in vitro metabolism of anthranilic acid, and particularly about the role of hydroxylations in it.

Of the indole skeleton derivatives, the hydroxylation has already been dealt with in vitro of skatole (HORNING et al., 1959), tryptamine (BRODIE et al., 1954), β-indoleacetic acid, tryptophane (DALGLIESH, 1955) and diethylgramine (MATKOVICS—MARIÁN, 1969a), and in vivo of tryptophane (MCILWAIN, 1966), indole (EICH—ROCHELMEYER, 1966) and dimethylgramine (DIGENIS, 1969a; DIGENIS 1969b).

With the exceptions of skatole, dimethylgramine and tryptamine, indole and all its derivatives listed have been made the subject of a systematic study of in vitro hydroxylation in order to obtain a deeper insight into the details and mechanisms of these hydroxylations.

Materials and Methods

All melting points were determined on a Böetius block and are uncorrected.

Ultraviolet spectra were obtained with a Spectromom 201 spectrophotometer (MOM, Budapest). Infrared spectra were determined in KBr on a Unicam SP 200 infrared spectrophotometer.

Kieselgel and Silica gel nach STAHL (Merck) and Reanal adsorbents were used in 0.25 mm layers on glass plates for the thin-layer-chromatography (TLC). For preparative purposes 1 mm layers were used.

Whatman No. 1 paper was used for the paper-chromatographic (PC) separation and identification of the hydroxy-anthranilic acids. In this case n-butanol saturated with 2.5 N HCl and butanol: galcial acetic acid: water (4 : 1 : 5) proved the best separatory systems (HILL et al.,

1966, VAN SUMERE et al., 1965; CEE-GASPARIC, 1966). In the separations of L-tryptophane and its hydroxy derivatives, however, besides the paper and PC systems mentioned a pyridine: water (8 : 2) mixture was used.

The TLC systems used in the separation of anthranilic acid, indole and its derivatives were the following:

a) Anthranilic acid and its hydroxy derivatives kere separated on Silica gel nach STAHL (Reanal) in chloroform : methanol : galcial acetic acid (75 : 20 : 5), chloroform : galcial acetic acid (95 : 5) and chloroform : galcial acetic acid : water (1 : 1 : 1) systems.

b) Indole and the hydroxyindoles were separated (as were the further substances) on Kicselgel G nach STAHL (Merck or Reanal); the systems used were benzene : acetone (9 : 1) and dichloromethane : ethyl acetate (65 : 35).

c) Chloroform : methanol : galcial acetic acid (75 : 20 : 5) was used for the separation of β-indole-acetic acid and its hydroxy derivatives.

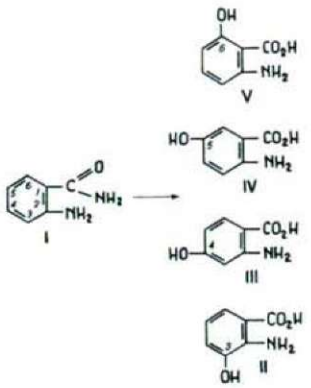
d) L-Tryptophane, diethylgramine and their hydroxy derivatives were spearated on the adsorbents mentioned in the order of listing. Isopropanol : ethyl acetate : 25% ammonia solution (5 : 3 : 2) or ethanol : 25% ammónia solution systems were employed.

Developer systems: van URK and PROHASZKA reagents and I_2 vapour (STAHL, 1967).

Anthranilic acid and 3-, 4-, 5- and 6-monohydroxynathranilic acids were purchased, or prepared and purified as described by STELT et al., (1952) and SHEEHAN (1948).

All the monohydroxyanthranilic acids could be isolated and separated in preparative quantities by thick-LC with the exception of 4-hydroxyanthranilic acid, and therefore III was prepared in a larger amount by a combination of the literature methods mentioned above.

4-Hydroxyanthranilic acid (III)

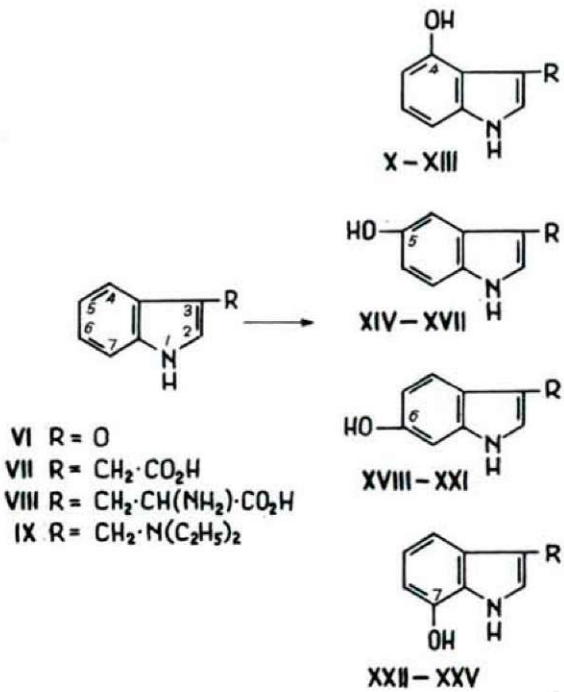


Mixture of 4-aminophenol (40 g) and ammonium carbonate (160 g) dissolved in distilled water (200 ml) was heated at 110 °C for 12 hours in an autoclave. The resulting suspension was filetered. The pH of the filtrate was adjusted to 3.2 with 2 N HCl (ca. 100 ml HCl was used), it was shaken at room temperature for 2 hours and again filtered. The clear solution was extracted with ether (3 × 200 ml), and the combined ether phases dried over anhydrous Na_2SO_4 and evaporated to dryness in vacuo. 20 ml 2 N HCl was added to the residue, it was again shaken for about 2 hours at room temperature and the pH controlled (it must be held at pH 3.2). After the repeated ether extraction the ether phase was washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness in vacuo to give a crystalline powder. Recrystallisation from water gave III with m. p. 161°C. (Found: C, 54.96; H, 4.64; N, 9.25%; $C_7H_7O_3N$ requires C, 54.91; H, 4.61; N, 9.15%.).

Hydroxyindoles (X, XIV, XVIII, XXII)

These were prepared as follows (EICH—ROCHELMEYER, 1966): 6 g VI was hydroxylated for 15 minutes at room temperature in the Fenton—Cier system. 6 g VI was dissolved with heating in 5 l 0.1 M phosphate buffer of

pH 7.2. 18.6 g EDTA disodium salt, 13.9 g $FeSO_4 \cdot 7H_2O$, 4.4 g ascorbic acid and finally 11.3 ml 3% H_2O_2 were added to the solution with shaking. After the reaction the aqueous filtrate was extracted with 3×200 ml petroleum ether, then with 4×200 ml peroxide-free ether; the extracts were dried with anhydrous Na_2SO_4, filtered and evaporated in an atmosphere of N_2. The product was 1.5 g mixed hydroxyindoles which were separated by thick—LC or on a column after acetylation, and identified.



Acetoxyindoles

Preparation (CROMARTLE—HARLEY, 1952): The above 1.5 g mixed hydroxiyindole residue was dissolved in 150 ml of a mixture of acetic anhydride:pyridine (4:6) and the solution left to stand at room temperature for about 4 hours. The isolation and separation were effected by the usual working-up procedure of acetylation. Finally, after the chromatographic separation the pure hydroxyindoles were prepared by the hydrolysis (BEER et al., 1948) with ammonia solution of methanolic solutions of the acetoxyindoles.

Hydroxyindoleacetic acids (XV, XVII)

Of the hydroxyindoleacetic acids only the 5- (XV) and 7-monohydroxyindoleacetic acids (XVII) are known. During the hydroxylation experiments these two hydroxy derivatives were formed and identified by PC on the basis of their R_f values (DALGLIESH, 1955).

Hydroxytryptophanes (XII, XVI, XX, XIV)

These were isolated and separated as described earlier (EICH—ROCHELMEYER, 1966).

Hydroxydiethylgramine (XIII, XVII, XXI, XXV)

These were prepared from the acetoxyindoles by Mannich condensation (MATKOVICS—MARIÁN, 1969a). 1.4 g acetoxyindole was dissolved in 10 ml dry dioxan and a mixture of 12 ml dioxan: galcial acetic acid (1:1), 0.83 ml 37% formaldehyde and 2.7 ml diethylamine was added dropwise during 15 minutes with ice cooling. After a further 2 hours in an ice bath the solution was allowed to attain room temperature slowly and to stand at room temperature for 1 days. 80 ml water was then added. A brown precipitate, a mixture of the acetoxy-diethylgramines, separated out and was filtered off. The diethylgramines formed from the indole residue were precipitated from the alkaline solution.

The following step in the synthesis of the hydroxydiethylgramines was the hydrolysis of the acetoxydiethylgramines. The earlier-mentioned filtered-off precipitate (ca. 0.3 g) was dissolved in 10 ml methanolic ammonia with cooling; the solution was allowed to stand for 1 day in an ice-box and then evaporated in vacuo to give a mixture of the hydroxyethylgramines. The hydroxyethylgramines were dissolved out of the residue with ether, and the pure materials could be obtained by thick—LC. The already-mentioned isopropanol: ethyl acetate: 25% ammonia solution (5:3:2) mixture was used for the separation and the pure substances were dissolved from the TLC adsorbent with ether. The analysis of the 4-, 5-, 6- and 7-monohydroxyethylgramine isomers: $C_{13}H_{18}N_2O$ requires C, 71.52; H, 8.31; N, 12.83; %. 4-Hydroxyethylgramine (XIII) had m. p. 150°C (found: C, 71.54; H, 8.35; N, 12.89%); 5-hydroxyethylgramine (XVII) had m. p. 168°C (found: C, 71.50; H, 8.34; N, 13.05 %; 6-hydroxyethylgramine had m. p. 133°C (found: C, 71.55; H, 8.33; N, 13.10 %); 7-hydroxyethylgramine had m. p. 108°C (found: C, 71.55; H, 8.30; N, 12.95 %).

Hydroxytryptophanes (XVIII, XXVI) (EICH—ROCHELMEYER, 1966).

Of the monophydroxytryptophanes only the 5- and 7-monohydroxytryptophanes formed. These were identified by PC as described by DALGLIESH (1955) and by TLC under conditions given above.

The different hydroxylation systems studied can be classified into three main groups:

A. Physical hydroxylation systems

UV irradiation was carried out for 24 hours with a home-made UV lamp at a distance of 15 cm.

(i). 1.0 mmole substrate was used in aqueous solution. (In allother cases the amount of substrate remained unchanged.)

(ii; iii). The pH of a solution of 1.0 mmole $FeCl_3$, Na_2EDTA and substrate in 100 ml distilled water was adjusted to 6.0 or 7.0.

(iv). 0.2 mmole H_2O_2 and the substrate in 100 ml water (BAXENDALE—MAGEE, 1954; BOYLAND—SIMS, 1953).

B. Chemical hydroxylation system
(ERICH—ROCHELMEYER, 1966.)

(i) Fenton system (FENTON, 1894): 3.0 mmole $FeSO_4 \cdot 7H_2O$, 6.0 mmole H_2O_2 and 3.0 mmole substrate in 100 ml 0.4 N H_2SO_4. Reaction time: 30—60 minutes.

(ii). Modified Fenton system (MATKOVICS—MARIÁN, 1969b): 1.0 mmole $FeSO_4 \cdot 7H_2O$, 1.0 mmole Na_2EDTA, 2.0 mmole H_2O_2 and 2.0 mmole substrate were dissolved in 100 ml 0.1 M phosphate buffer solution of pH 7.2. Reaction time: 30—60 minutes.

(iii). Fenton—Cier system (MATKOVICS—MARIÁN, 1969b); 1.0 mmole Na_2EDTA, 0.5 mmole L-ascorbic acid, 2.0 mmole H_2O_2 and 2.0 mmole substrate were dissolved in 100 ml 0.1 M phosphate solution of pH 7.2. Reaction time: 15—30 minutes.

(iv). Udenfriend system (BRODIE et al., 1954; UDENFRIEND et al., 1954): 0.5 mmole $FeSO_4 \cdot 7H_2O$, 2.6 mmole Na_2EDTA, 5.6 mmole L-ascorbic acid and 2.0 mmole substrate were dissolved in 100 ml 0.1 M phosphate buffer solution pH 6.7. Reaction time (with shaking): 2—24 hours.

In general the incubation was carried out at room temperature or at 37°C. A control was always prepared with the omission of the reagents. After the given reaction time the substrances were extracted with specific solvents reported in the literature, and the extracts were dried and evacuated in vacuo in a N_2 atmosphere.

C. Incubation experiments with adrenal tissue

Fresh cattle adrenal was used for incubation purposes; cut into thin slices it was placed into KREBS—RINGER phosphate buffer and incubated in varius ways.

(i). Adrenal slices of 10.0 g weight are placed in 100 ml KREBS—RINGER phosphate buffer of pH 7.0 containing 10^{-2} M substrate.

(ii). The same as (i) but the solution also contains 5×10^{-2} M L-ascorbic acid.

(iii). To 10.0 g adrenal slices in 100 ml KREBS—RINGER phosphate buffer as above are added 10^{-4} M substrate, 10^{-3} M potassium fumarate and L-ascorbic acid, 0.001 g L-ascorbic oxidase and 2×10^{-5} M NADPH (the sodium salt of NADPH) Calbiochem, Los Angeles, Calif., USA was used.

The above incubation mixtures (i-iii) were prepared with 10 ml microsome and mitochondrium mixtures too, obtained from adrenal of 30 g weight by the method of SWEAT (1951) and diluted with 100 ml KREBS—RINGER phosphate buffer of pH 7.2.

The L-ascorbic acid oxidase was purified and determined by the method of TAUBER et al. (1953).

The incubations were carried out at 37°C under a constant O_2 flow. The reaction time was 5 hours. After incubation the proteins were precipitated with 20 ml 20% tricholoracetic acid, the precipitate was filtered off and the filtrate was extracted with various solvents depending on the incubated substance.

Identification was accomplished by examination and comparison of the physical properties and identical behaviour shown in several chromatographic systems and also the running together with known compounds. (We shall return to special considerations of the above-mentioned methods in the discussion of the results.)

Results

The hydroxylation of anthranilic acid (I) was carried out in all the systems and conditions listed. Of the systems A/i-iv under the conditions of A/iv we succeeded in detecting only hydroxylation; IV is formed in largest amount and II in only a chromatographically detectable amount.

Hydroxy derivatives could not be detected in the B/i system (FENTON system). The products obtained in the systems B/ii-iv are compared in Table 1, taking the amount of III as 100.

Table 1

System	II	III	IV	V
B/ii	291	100	182	traces
B/iii	172	100	105	traces
B/iv	220	100	149	traces

In the case of the incubations C/i-iii carried out with adrenal slices, mitochondria and microsomes, only II could be detected.

Figures 3 and 4 show two typical examples of TLC plates in the $CHCl_3$:MeOH:AcOH (75:20:5) system, on the one hand under different hydroxylation conditions, and on the other hand on a thick layer (Fig. 4).

The hydroxylation og indole (IV) led to the following results: In the A/i and A/iv systems, indigo, isatin and many still unidentified products were detected. Tn the A/ii and A/iii systems which also contained Fe^{3+}ions, in addition to the above products XVIII was identified.

The results obtained in the systems B/ii-iv (Tables II and III) were compared both with each other and with the data reported by EICH et al. (1966).

Here too no hydroxylation could be detected in the B/i system.

Our own results are compared in Table II, taking XXII as 100.

Table II

System	X	XIV	XVIII	XXII
B/ii	102	100	100	100
B/iii	101	120	101	100
B/iv	135	120	135	100

Table III gives a comparison of our own results weith literature data. Our results in the Udenfriend system (B/iv) agreed with the results of EICH et al. (1966), and only in the B/iii system was there a small difference.

Table III

System	X	XIV	XVIII	XXII
B/iii	24	28	25	23
Fenton-Cier*	25	33	25	17

* Data of EICH, ROCHELMEYER (1966)

(In the Table the relative ratios of the substances are compared.)

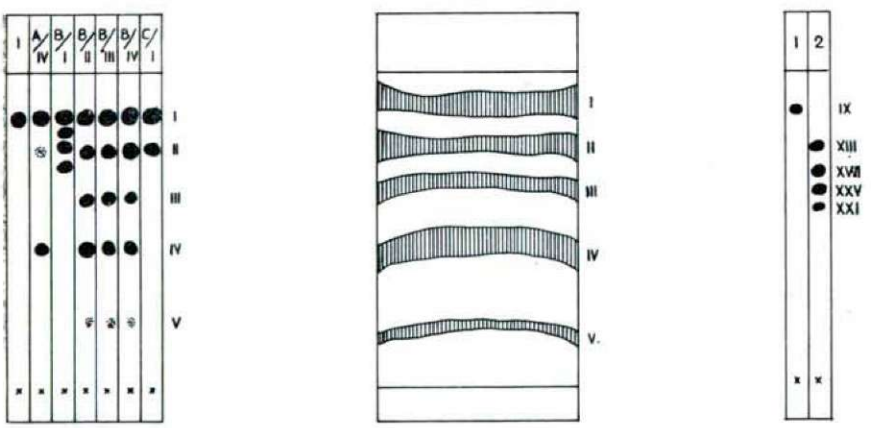
On incubation with adrenal slices under different conditions, 4-, 5-, 6- and 7-hydroxyindoles could be detected.

The hydroxylation of β-indoleacetic acid (VII). Of the A group irradiations, only under the conditions of A/iv could a small amount of 5-hydroxyindoleacetic acid (XV) be detected and identified by TLC.

In both the B/iii and B/iv systems, the formation of 5- and 7-hydroxyindoleacetic acids (XV and XXIII) could be detected. The ratio of the two products was 1.40:1.0 (XV:XXIII). Hydroxylation could not observed in the other two systems.

Under the conditions of C/i-iii, only small amounts of XV could be detected.

The hydroxylation of tryptophane (VIII) did not lead under any conditions to a great variation of the products.



Under the given conditions the irradiation did not lead to hydroxy derivatives. Similarly ineffective from the point of view of hydroxylation were the incubations B/i and B/ii. The main products in the systems B/iii and B/iv were XIV and XXIV, but the detection of the other two products was also possible by

TLC. For example, in the system B/iii, XVI (5-hydroxy-) and XXIV (7-hydroxytryptophane) were obtained in a ratio of 5:1.

Under the conditions of C/i-iii, only XVI and XXIV were detected in small amounts.

The hydroxylation of ethylgramine (IX) does not occur as a result of ultraviolet radiation. The system B/i does not hydroxylate IX. The systems B/ii, B/iii and B/iv all promote the formation of 4-, 5-, 6- and 7-hydroxyethylgramines. Of these methods, B/iii is the most effective, i. e. it leads to the highest transformation ratios. The isomers were separated by TLC (see Figure 5).

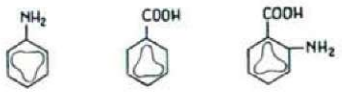
Hydroxyethylgramines could not be separated from adrenal incubates.

Discussion

The results are discussed in the above order, with stress laid on the common features which are suitable for the explanation of the happenings.

The ultimate aim of our study is the elaboration of a combination method of preparation of hydroxy derivatives which are difficult to obtain by chemical methods, thereby permitting the production of hydroxy derivatives in good yield.

A common feature of the physical (ultraviolet radiation) and chemical methods is the radical formation. The ensuing reaction of the hydroxy radicals with the aromatic system is influenced by the substituents in various ways (WILLIAMS, 1960). Of course, it must also be said that all four different hydroxy derivatives are formed in the B/i-iv systems, and hence only in their ratio can a definite difference be detected, although this difference is often very slight (see Table 2). The above can most simply be illustrated in the hydroxylation of anthranilic acid (I). The amino group, as a substituent of class I, primarily assists the entrance of a new electrophilic substituent in the two ortho and para positions. The carboxyl group, however, as a class II substituent facilitates the entry of the electrophilic group in the two meta positions (see the Figures). If the two activation sites are compared, it is seen that electrophilic substitution of anthranilic acid primarily favours the 3 and 5 C atoms. Of the hydroxy derivatives, however, 4-hydroxyanthranilic acid (III) is formed in significant amount. This thus supports the conception of a radical reaction mechanism.



Still further evidence for the radical mechanism is given by the ratios between the hydroxy substitutions observed with indole and its derivatives. Radicals are similarly formed as a result of ultraviolet radiation, but is often observed with hydroxy derivatives of indole; primarily the 3-hydroxyindole necessary for the formation of indigo is obtained, and this is transformed to indigo by the action of air.

With chemical methods in the case of indole and its derivatives which we have studied, the hydroxy derivatives formed are much more uniformly distributed than was observed for anthranilic acid. It is found, considering the radical mechanism, that the „quantitative" ratio and nature of the hydroxy derivatives depend to a large extent on the effect of the radical bonded to the indole skeleton at C—3. It has already been mentioned that harmonic chemical hydroxylation is observed with indole (relatively uniform monohydroxy derivative distribution), but with indoleacetic acid and tryptophane hydrpxylation occurs only at C—5 and C—7 althrough the chemical conditions are the same.

In general, differences can otherwise be observed between the chemical systems listed in the intensity of hydroxylation. However, this intensity can not be interconnected with the electrophilic strengths of the systems because these change in the order:

B/i B/ii B/iv B/iii

In practice, however, it was observed that in contrast to the electrophilic strengths of the different systems the transformations were in the following order of intensity from a qualitative viewpoint:

B/iii B/iv B/ii B/i

The chemical hydroxylation is again uniform for ethylgramine hydrochloride, altrough in this case the percentage transformation is of very small.

The incubation carried out with adrenal slices and mitochondria under different conditions resulted in only some of the already-reported derivatives. The products formed here too point to the radical mechanism, and in our opinion can not be regarded as the consequences of specific enzyme effects but for example as non-specific hydroxylating action, e. g. the side-effects of peroxidase. These unsolved questions are being studied further.

Summary

1. Studies were made of the in vitro hydroxylation of anthranilic acid (I), indole (VI), indoleacetic acid (VII), trytophane (VIII) and diethylgramine (IX) in different physiological and physical hydroxylation systems.

2. The products were isolated and compared with known or newly synthesized compounds.

3. In different hydroxylation systems different products could be formed.

4. Some of the hydroxylation systems were selective in the hydroxylation of different substrates.

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