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The Oxidation of Tryptophan and Some Related Compounds with Persulphate

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The oxidation of tryptophan and some related compounds with persulphate has been studied in the light of recent knowledge of the metabolism of this amino acid (cf. Dalgliesh, 1951). The compounds related to tryptophan which have been oxidized include kynurenine, 3-indolylacetic acid and indole, and the oxidation of anthranilic acid has been re-examined. A number of chemical oxidations of tryptophan have been reported, and the present work the action of alkaline persulphate on DL-tryptophan, under conditions similar to those previously described for the persulphate oxidation of aromatic amines (Boyland, Manson & Sims, 1953; Boyland & Sims, 1954), has led to the formation of a number of acid-labile and other products (see Fig. 1), some of which have either been isolated or identified by means of paper chromatography.

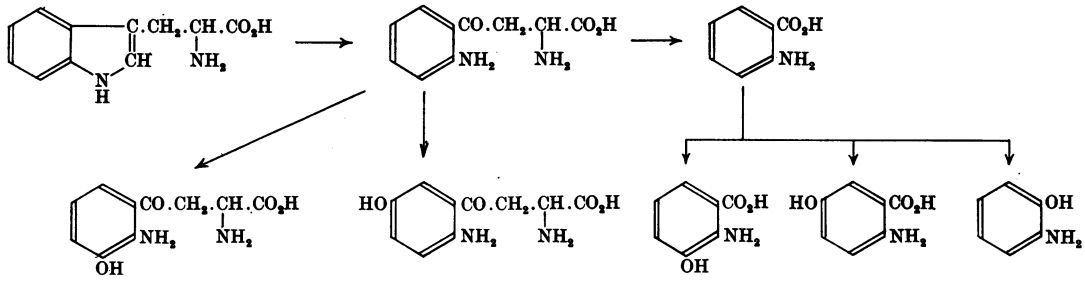

Fig. 1. Oxidation of DL-tryptophan with persulphate.

usually they involve either attack on the side chain or fission of the pyrrole ring. With ferric chloride, for example, 3-indolylaldehyde is formed (Hopkins & Cole, 1903; Ellinger, 1906), and ozonolysis yields *N'*-formylkynurenine (Witkop & Graser, 1944; Knox & Mehler, 1950). Peracetic acid, however, hydroxylates L-tryptophan in the 2-position to yield β-3-oxindolylalanine (Witkop, 1947); Dalgliesh (1954) has shown that with the ascorbic acid-hydroxylating system of Udenfriend, Clark, Axelrod & Brodie (1954) and Brodie, Axelrod, Shore & Udenfriend (1954), 5- and probably also 7-hydroxytryptophan are formed. In

EXPERIMENTAL

Melting points are uncorrected.

Paper chromatography. Descending chromatograms on Whatman no. 1 chromatography paper were developed with the organic phase of *n*-butanol–acetic acid–water (4:1:5, by vol.) (Partridge, 1946). The oxidation products were characterized on the papers by examination under a Hanovia Chromatolite ultraviolet lamp, and by spraying the papers (*a*) with 5% *p*-dimethylaminobenzaldehyde in 10% aqueous ethanol containing 2·5% HCl (Ehrlich's reagent), (*b*) with 0·4% ninhydrin in *n*-butanol saturated with water (the colours produced were sometimes slow in appearing, so that the chromatograms were examined 2 hr.

Table 1. *The colour reactions given by the acid-labile persulphate oxidation products after chromatography*

Methods are given in the Experimental section.

Probable identity	R_F	Fluorescence	Colour reactions: Ehrlich's reagent: Immediate	Ehrlich's reagent: After 2 hr.	Ninhydrin	$HCl + NaNO_2$	$HCl + NaNO_2$ hexylresorcinol
o-Aminophenyl sulphate	0·32	—	Yellow	Yellow	—	Yellow	Scarlet
Sulphuric ester of 3-hydroxyanthranilic acid	0·26	Blue	Yellow	Pink	—	—	Yellow
Sulphuric ester of 5-hydroxyanthranilic acid*	0·13	Blue	Orange	Orange	—	—	Yellow
Sulphuric ester of 3-hydroxy-DL-kynurenine*	0·04	Blue-green	—	Pink	Reddish purple†	Yellow	Pink
Sulphuric ester of 5-hydroxy-DL-kynurenine*	0·02	Blue-green	Orange-yellow	Orange-yellow	Purple†	Yellow	Pink
Indoxyl sulphate	0·25	Violet	Brown	Blue†	—	Grey	Yellow, then blue

* These esters could not be compared directly with the authentic compounds and their structures are inferred from the nature of their hydrolysis products.
† After 24 hr.

Table 2. *The colour reactions given by the persulphate oxidation products of tryptophan after acid hydrolysis and chromatography*

Methods are given in the Experimental section.

Product	R_F	Fluorescence	Colour reactions: Ehrlich's reagent	Ninhydrin: After 2 hr.	Ninhydrin: After 24 hr.	$HCl + NaNO_2$	$HCl + NaNO_2$ hexylresorcinol
o-Aminophenol	0·66	—	Yellow	—	—	Brown	Crimson
3-Hydroxyanthranilic acid	0·80	Blue	Yellow, turning pink	—	—	Greenish	Reddish brown
5-Hydroxyanthranilic acid	0·52	Light blue	Orange-yellow	—	—	—	Scarlet
3-Hydroxy-DL-kynurenine	0·20	Green	Pink	Pale yellow	Brown-purple	Brown	Reddish brown
5-Hydroxy-DL-kynurenine	0·10	Yellowish green	Orange-pink	Pale yellow	Grey-purple	—	Pink
DL-Kynurenine	0·23* 0·27	Light blue	Orange	Pale yellow	Purple	—	Yellow
Anthranilic acid	0·85	Violet	Deep yellow	—	—	—	Orange-yellow

* Resolved into D- and L-forms.

and again 24 hr. after spraying), and (*c*) with 2·5% aqueous $NaNO_2$ followed by N-HCl, noting any colour produced, and finally spraying with 0·5% hexylresorcinol in 2N-NaOH (cf. Booth, Boyland & Manson, 1955). Where possible the oxidation products were compared directly on the paper chromatograms with the reference compounds described below. The properties of the acid-labile compounds are listed in Table 1 and those of the phenols derived from them by acid hydrolysis in Table 2.

Reference compounds. DL-Kynurenine, anthranilic acid, 3-hydroxyanthranilic acid and *o*-aminophenol were obtained from commercial sources, and 5-hydroxyanthranilic acid was prepared by the method of Limpricht (1891). *o*-Aminophenyl sulphate was prepared from *o*-nitrophenol as described by Burkhardt & Wood (1929) and the sulphuric ester of 3-hydroxyanthranilic acid by the method of Boyland & Sims (1954). The latter ester, which was purified by several precipitations with 2N-HCl from its solutions in saturated aqueous $NaHCO_3$, formed one spot on paper chromatograms and yielded only 3-hydroxyanthranilic acid and inorganic sulphate after hydrolysis with hot 2N-HCl. 3- and 5-Hydroxy-DL-kynurenine were the gift of Professor A. Butenandt. These compounds had been prepared by the methods of Butenandt & Hellmann (1950) and Butenandt, Schulz & Hanser (1953) respectively. The *p*-toluidine salt of indoxyl sulphate obtained from Dr S. J. Holt had been prepared from the naturally occurring ester.

Persulphate oxidations. DL-Tryptophan (20 g.), DL-kynurenine (2·5 g.) or 3-indolylacetic acid (10 g.) was dissolved in aqueous 2N-KOH (20% excess of the theoretical

amount) and treated with aqueous $K_2S_2O_8$ (10% excess of the theoretical amount) added dropwise at room temp. over 6 hr. with continuous stirring. Some experiments were carried out with ice cooling, but this had no effect on the course of the reaction. Indole (10 g.) in 50% (v/v) aqueous acetone containing 2N-KOH (20% in excess of the theoretical amount) was similarly treated. The mixtures were kept overnight, evaporated to about 100 ml. under reduced pressure and made just acid with 10N-H_2SO_4. The dark flocculent precipitates which usually separated were filtered off and discarded. The filtrates were washed continuously with ether for some hours, and the aqueous layers were made alkaline with 2N-KOH and evaporated to dryness under reduced pressure; the residues were extracted several times with boiling methanol. The ethereal and methanolic extracts were treated as described below.

Oxidation of tryptophan. (*a*) *Ethereal extract.* A 2 ml. portion of the extract was evaporated and the residue was mixed with a few drops of 2N-HCl and a little magnesium powder was added. The mixture was filtered, and to the filtrate was added 75% H_2SO_4 (3 ml.) and a little chromotropic acid. The mixture was heated to 60°, and a violet-pink colour appeared, indicating the presence of formic acid in the ethereal extract (cf. Feigl, 1954).

The rest of the ethereal extract was evaporated to dryness, the residue was dissolved in saturated aqueous $NaHCO_3$ (2 ml.) and the solution was washed with ether (2 × 10 ml.). The aqueous layer was acidified with 2N-H_2SO_4 and extracted with ether. The ether was removed and the residue was recrystallized several times from ethanol to yield anthranilic acid (28 mg.), m.p. and mixed m.p. 142–144°. The identity of the acid was confirmed by paper chromatography.

(*b*) *The methanolic extract.* This was evaporated to dryness under reduced pressure to yield a brown gum which set to a brown resin. Examination of this resin on paper chromatograms showed the presence of unchanged DL-tryptophan together with a large amount of DL-kynurenine and small amounts of the products (except indoxyl sulphate) whose properties are listed in Table 1. When a little of the resin was heated to 100° with a few drops of 2N-HCl and the mixture examined on paper chromatograms, the phenols listed in Table 2 were detected. Among the products of the reaction detected on paper chromatograms which could not be identified was one forming spots (R_F 0·04) which were non-fluorescent, but gave a purple colour with ninhydrin. When the resin was treated with hot 2N-HCl this substance could no longer be detected, but a second non-fluorescent substance of R_F 0·16 was present, which gave a purple colour with ninhydrin.

A column 4 cm. in diameter and 20 cm. long was packed with Hyflo Super-Cel (Johns-Manville Co. Ltd., London, S.W.1) containing 25% (w/v) of water as a stationary phase, using a Martin packer (Randall & Martin, 1949). A little of the resin was dissolved in water (1 ml.) and added to a layer (2 cm.) of dry 'Super-Cel' which had been packed on the top of the column. The moving phase [amyl alcohol (British Standard 696)–*cyclo*hexane, 1:3, by vol.] was added and a pressure of 100 mm. Hg applied to the top of the column. The eluate was collected in 10 ml. fractions and these were examined in ultraviolet light. The fractions comprising 110–160 ml. of the eluate were mixed and concentrated, when DL-kynurenine, m.p. and mixed m.p. 205–210° (decomp.), was deposited as a yellow solid. (Found: N, 13·1. Calc. for $C_{10}H_{12}O_3N_2$: N, 13·5%.) The compound was indistinguishable from authentic DL-kynurenine on paper chromatograms.

Oxidation of DL-*kynurenine.* (*a*) *The ethereal extract.* Paper chromatography showed the presence of a compound with the same properties as anthranilic acid.

(*b*) *The methanolic extract.* This was evaporated to dryness under reduced pressure to leave a yellow solid. Examination of the solid on paper chromatograms revealed the presence of the products (except indoxyl sulphate) listed in Table 1. When the solid was heated to 100° for 15 min. with 2N-HCl these products could no longer be detected on paper chromatograms, but spots characteristic of the phenols listed in Table 2 were now present.

The solid was dissolved in water (100 ml.) and passed through a column 2 cm. in diameter and 15 cm. long of the acid form of the ion-exchange resin, Zeo-Karb 225. A further 100 ml. of water was passed through the column and the combined eluates were evaporated to dryness under reduced pressure. The solid residue was dissolved in the minimum of water and transferred on to a Hyflo Super-Cel column prepared as before. An equilibrated moving phase of amyl alcohol (B.S. 696)–light petroleum (b.p. 80–100°) (4:1, by vol.) was passed through the column, and the eluted fractions (*a*) 0–100 ml., (*b*) 200–300 ml. and (*c*) 300–400 ml. were collected separately and evaporated to dryness.

Fraction (*a*) formed a colourless powder which was shown by paper chromatography to contain a compound with the same properties as the sulphuric ester of 3-hydroxyanthranilic acid, together with small amounts of *o*-aminophenyl sulphate and a substance identical with one obtained in the anthranilic acid oxidation (see below).

Fraction (*b*) was dissolved in hot *n*-octanol (5 ml.) and allowed to crystallize in a vacuum desiccator; a small amount of pale-yellow needles separated, which were considered to be the sulphuric ester of 3-hydroxy-DL-kynurenine since the compound yielded only one spot on paper chromatograms and, after hydrolysis with hot 2N-HCl or with Taka diastase, only 3-hydroxy-DL-kynurenine could be detected on paper chromatograms. Inorganic sulphate was present in the hydrolysed solution.

Fraction (*c*) was a mixture which formed two spots on paper chromatograms, one of which was identical with that of the compound just described. When the mixture was hydrolysed as before, the hydrolysis product yielded two spots on paper chromatograms, identical with those formed by authentic 3- and 5-hydroxy-DL-kynurenine. It seems likely therefore that the second component of this fraction is the sulphuric ester of 5-hydroxy-DL-kynurenine.

The ion-exchange resin was treated with 2N-HCl (250 ml.) and the eluate was evaporated to dryness under reduced pressure. The solid was taken up in a little water and transferred to a column of Hyflo Super-Cel similar to that described above, but with a moving phase consisting of amyl alcohol–light petroleum (b.p. 80–100°) (1:9, by vol.). The eluted fraction 50–120 ml. yielded anthranilic acid, m.p. and mixed m.p. 142–143°. The eluted fraction 350–450 ml. yielded 3-hydroxyanthranilic acid, decomp. 210–240°. (Found: N, 9·3. Calc. for $C_7H_7O_3N$: N, 9·15%.) This compound formed one spot on a paper chromatogram identical with that of authentic 3-hydroxyanthranilic acid.

Oxidation of 3-*indolylacetic acid.* The ethereal extract was shown by paper chromatography to contain anthranilic acid.

The methanolic extract was examined by paper chromatography. A number of substances were present, but the only ones recognized had properties identical with the acid-labile compounds which were also obtained in the anthranilic acid oxidation described below.

Oxidation of indole. The ethereal extract was shown by paper chromatography to contain a substance indistinguishable from anthranilic acid.

The methanolic extract was evaporated to small volume under reduced pressure and allowed to crystallize. Potassium indoxyl sulphate (3·8 g.) separated; from aqueous ethanol it formed light brown plates. (Found: C, 38·5; H, 2·8; N, 5·3; S, 13·0. Calc. for $C_8H_6O_4NSK$: C, 38·2; H, 2·4; N, 5·6; S, 12·75%.) The *p*-toluidine salt separated from water in pale-blue plates. (Found: N, 8·9. Calc. for $C_{15}H_{16}O_4N_2S$: N, 8·75%.) The infrared spectrum of the *p*-toluidine salt was identical with that of the *p*-toluidine salt of the naturally occurring indoxyl sulphate.

The mother liquors were examined by paper chromatography and contained, in addition to indoxyl sulphate, the acid-labile products which were also formed in the anthranilic acid oxidation.

Oxidation of anthranilic acid. The oxidation of anthranilic acid with persulphate already described (Boyland & Sims, 1954) was re-examined and the mixture of acid-labile products which was isolated was examined by paper chromatography. The principal component of the mixture was the sulphuric ester of 3-hydroxyanthranilic acid, but a spot with properties identical with those of *o*-aminophenyl sulphate was also present. A third substance which formed spots on the chromatograms of the unhydrolysed mixture could no longer be detected by this means after the mixture had been heated with 2N-HCl for 15 min. These chromatograms now showed spots which could not be distinguished from authentic 3- and 5-hydroxyanthranilic acid and *o*-aminophenol respectively. It is considered likely, therefore, that the unknown acid-labile substance is the sulphuric ester of 5-hydroxyanthranilic acid.

The mixture of esters (500 mg.) was heated to 100° with 2N-HCl for 15 min. and the solution was treated with a small excess of solid $NaHCO_3$ and extracted with ether. Evaporation of the ether afforded *o*-aminophenol (10 mg.), which, after crystallization from water, had m.p. and mixed m.p. 173–174°.

DISCUSSION

Alkaline persulphate appears to attack tryptophan at the 2:3-bond of the pyrrole ring. The first product of this reaction may be the disulphuric ester of 2:3-dihydro-2:3-dihydroxytryptophan, which would yield the free dihydroxy compound on acid hydrolysis. It is possible that the ester and the free hydroxy compounds are the substances with R_F values 0·04 and 0·16 respectively, which were detected in the tryptophan oxidation. The substance of R_F 0·16 is not β-3-oxindolylalanine, neither is it one of the hydroxytryptophans formed in the ascorbic acid oxidation system of Brodie *et al.* (1954) and of Udenfriend *et al.* (1954). 2:3-Dihydro-2:3-dihydroxytryptophan, it has been suggested (Knox & Mehler, 1950; Dalgliesh, Knox & Neuberger, 1951), is a possible intermediate in tryptophan metabolism.

Fission of the 2:3-bond of tryptophan would lead initially to the formation of *N'*-formylkynurenine. This has not been detected, but both formic acid and kynurenine are present in the reaction mixture. Only small amounts of the sulphuric esters derived from the further oxidation of tryptophan were detected, possibly because *N'*-formylkynurenine is only slowly hydrolysed during the reaction; acetyl derivatives of aromatic amines do not react with persulphate as do the parent amines (Boyland & Sims, 1954).

The sulphuric ester of 3-hydroxy-DL-kynurenine gives similar colours on paper chromatograms to those given by a substance which Dalgliesh (1952) detected in the urine of rats fed with supplementary tryptophan. That the substance was the sulphuric ester of 3-hydroxy-L-kynurenine was adduced by Dalgliesh from a consideration of some of its physical properties, together with the formation of 3-hydroxy-L-kynurenine and inorganic sulphate on acid hydrolysis.

In the oxidations of DL-kynurenine and anthranilic acid the substitution of sulphuric ester groups in positions both *ortho* and *para* to the amino groups is unexpected, in view of the fact that *para* substitution has not been detected in the persulphate oxidation of other aromatic amines (Boyland *et al.* 1953; Boyland & Sims, 1954). Both these amines have carbonyl groups *ortho* to the amino groups, and it is possible that the *meta*-directing influence of the carbonyl group has some effect on the position of substitution of the ester group. *o*-Aminoacetophenone also yielded two acid-labile products with persulphate, which are apparently the sulphuric esters of 2-amino-3- and 2-amino-5-hydroxyacetophenone, although their identity has not been conclusively established. These products were not detected among the products of the persulphate oxidation of tryptophan.

SUMMARY

1. The actions of alkaline persulphate on DL-tryptophan, DL-kynurenine, 3-indolylacetic acid and indole have been examined, and its action on anthranilic acid has been re-examined. A number of products have been isolated or identified by means of paper chromatography.

2. All the above compounds yielded anthranilic acid, the sulphuric esters of 3-hydroxyanthranilic acid and *o*-aminophenol, and a product which is probably the sulphuric ester of 5-hydroxyanthranilic acid.

3. DL-Tryptophan also yielded formic acid and DL-kynurenine, together with products which are considered to be the sulphuric esters of 3- and 5-hydroxy-DL-kynurenine. These products were obtained in better yield from the oxidation of DL-kynurenine itself.

4. Indole gave indoxyl sulphate in good yield, identical with the naturally occurring ester.

We thank Professor A. Butenandt for the gift of synthetic 3- and 5-hydroxykynurenine, Dr C. E. Dalgliesh for the gift of some DL-kynurenine and Dr S. J. Holt for the gift of the *p*-toluidine salt of authentic indoxyl sulphate. We also thank Mr S. F. D. Orr for his help in carrying out the infra-red absorption spectra. Analyses are by Mr F. H. Oliver of the Organic Chemistry Department, Imperial College of Science and Technology. This work has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research, Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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A Collimated Windowless Geiger Counter for Scanning Chromatograms

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The scanning of paper chromatograms and electrophoresis strips is a common requirement in biochemical laboratories using isotopes. A number of automatic apparatuses for doing this are available commercially in this country and use the standard E.H.M. (G.E.C. Research Laboratories) end-window counters. Collimation of the E.H.M.2.S. end-window counter to give a slit 5 mm. wide (which is desirable for analysing protein electrophoresis strips) reduces the sensitivity of such a counter by about 60%. A windowless counter with suitable geometry increases the sensitivity about three times. The counter described below was designed to combine the sensitivity of a windowless counter with narrow collimation and a low background.

Although a counter operating in the proportional region will tolerate contamination of counting gas with air, proportional counting necessitates additional expensive equipment which is not always available. A counter operating in the Geiger region (coupled to a standard rate meter) does not tolerate any contamination of counting gas with air, and an effective seal is therefore necessary.

CONSTRUCTION

The dimensions and assembly of the counter are shown in Fig. 1. The counter body is made from drawn brass tubing 1·8 cm. in internal diameter, 2·5 cm. in external diameter and 8 cm. long, milled externally to give a flat surface 1·6 cm. wide; a window 3·0 cm. × 0·5 cm. is cut out in the middle of this flat surface. All surfaces and corners on the inside of the counter are polished smooth. The ends of the counter are of brass plate into which insulating polythene plugs are set. The anode (tungsten wire 0·002 in. in diameter) is shielded by brass sleeves, leaving an effective anode length of 2 cm. in the centre. A lead castle surrounds the upper surface and sides and projects 3 cm. beyond the ends of the counter. The castle is mounted on a brass plate which rests on collars surrounding the four locating studs screwed into the lead base.

An effective air-counting gas seal is achieved by the flat surface around the counter window resting on the paper strip, which is mounted on a Tufnol (Tufnol Ltd., Perry Barr, Birmingham, 22B) strip