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The Nonenzymatic Conversion of Tyrosine into Mono- and Dihydroxyindoles*

Meir Wilchek,† Thomas Spande, George Milne, and Bernhard Witkop

ABSTRACT: The reaction of methyl or ethyl tyrosinate, tyrosineamide, and *N*-methylamide in dilute aqueous acetic acid with *N*-bromosuccinimide (NBS) or bromine results first in rapid *o,o'*-bromination and a commensurate increase of the absorption of the phenolic chromophore at 275–280 mμ. A third mole of NBS produces the labile tribromodienone (*e.g.*, XVIII, λ_{max} 270 mμ) which can either revert to the dibromotyrosine level or rearrange to a 5,7-dibromo-6-hydroxyindole (X) resulting from intramolecular Michael addition of the amino group of the tyrosine side chain to the dienone system. At pH 6 this rearrangement proceeds with loss of two bromines to a 5,6-dihydroxy-7-bromoindole (XXV).

Compound XXV was obtained also by the action of NBS on the ethyl ester (XXX) of 3,4-dihydroxyphenylalanine (dopa) with ethyl 5,6-dihydroxy-7-bromo-2,3-dihydroindole-2-carboxylate (XXXI, isolated as the 5,6-diacetate XXXII) as an intermediate or by-product which is spontaneously dehydrogenated to the corresponding indole XXXIII on standing in alkaline solution. Oxidation of dopa ethyl ester XXX by NBS in addition gave a bromine-free product which was isolated as the *O,O,N*-triacetate XXXIV. Mechanisms for these transformations which make possible for the first time the nonenzymatic conversion of tyrosine derivatives into 6-hydroxy- and 5,6-dihydroxyindoles are discussed.

The action of *N*-bromosuccinimide (NBS)[1] on tyrosine-containing peptides and proteins normally results in the scission of the carboxyl amide bond of tyrosine and formation of a spirodienonelactone (V) (Scheme I) with the concomitant release of a new NH_2-terminal peptide (Schmir *et al.*, 1959; Wilson and Cohen, 1963a). This oxidative as well as electrolytic peptide cleavage (Iwasaki *et al.*, 1963; Farber and Cohen, 1966) has been useful in determining or "auditing" primary sequences of proteins independent of enzymatic procedures (Witkop, 1961; Ramachandran and Witkop, 1967). The reaction pathway with NBS has been shown to involve two discrete steps: (i) rapid consumption of 2 moles of NBS and *ortho* bromination of the phenolic moiety to give the 3,5-dibromotyrosyl residue (I); (ii) slower consumption of a third mole of NBS to afford the comparatively stable spirodienonelactone (V) which may arise by either stepwise or concerted nucleophilic attack of the amide carbonyl group of tyrosine upon a labile tribromo intermediate, such as II or III, to yield the unstable iminolactone IV (*cf.* Schmir and Cunningham, 1965; Cunningham and Schmir, 1966), which undergoes spontaneous hydrolysis to the final products.

As judged by ultraviolet spectroscopy the formation

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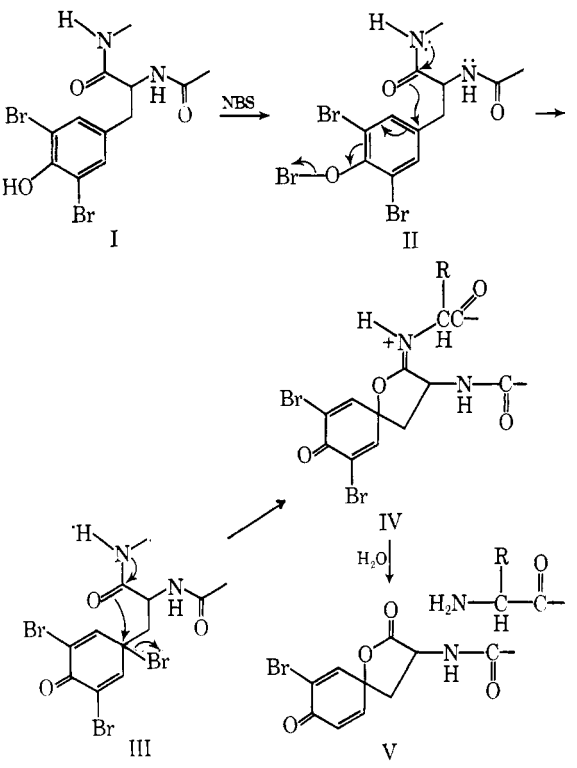
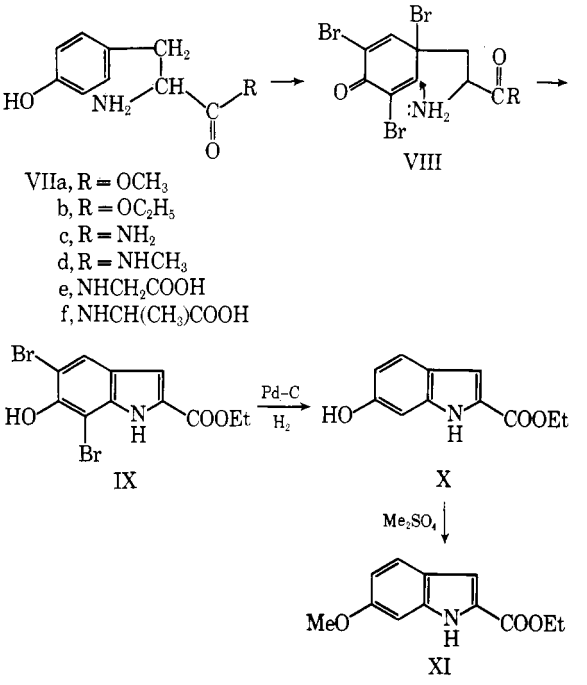
[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: NBS, *N*-bromosuccinimide; dopa, 3,4-dihydroxyphenylalanine.

SCHEME I

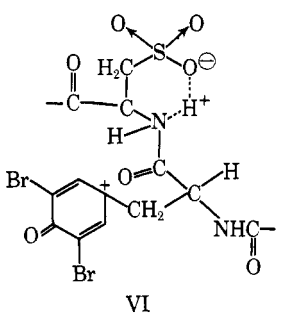


SCHEME II



of the spirodienonelactone system in tyrosine-containing proteins, *e.g.*, bovine ribonuclease, is nearly quantitative. However, the yields of new NH_2-terminal amino acids only range from 30 to 60% (Wilson and Cohen, 1963a) when the reaction is carried out under optimal cleavage conditions, namely, acidic medium and sufficient NBS to provide enough oxidant for the oxidation of other NBS-sensitive residues, such as tryptophan, histidine, cystine, and lysine.

No cleavage was observed when the residue following tyrosine was either cystine or cysteic acid (Wilson and Cohen, 1963b). Under the conditions of the cleavage reaction, NBS converts cystine into cysteic acid and it is this strongly acidic sulfonic acid group which presumably prevents the participation leading to cleavage, by protonation of the amide nitrogen as hypothesized in VI.



The cleavage reaction also fails when tyrosine is the NH_2-terminal residue in di-, tri-, and tetrapeptides as well as in tryptic fragments from proteins (Wilchek and Witkop, 1967). Since NH_2-terminal tyrosine occurs rarely in proteins (Dayhoff *et al.*, 1965), failure to cleave in this instance does not limit the usefulness of the method.

We have now looked into the question why NH_2-terminal tyrosine peptides fail to show the normal cleavage reaction with NBS (Wilchek *et al.*, 1967). The NH_2-terminal tyrosyl amino group has a pK_a of 7.5–8.2 (Edelhoch *et al.*, 1967) and would be fully protonated at the pH employed in the cleavage reaction. The proximity of such an ammonium group to the amide carbonyl could interfere with the participation and cleavage (Isoe and Cohen, 1968). In addition, the free α-amino group of tyrosine could conceivably be oxidatively deaminated to an α-keto group in a manner similar to the conversion of primary amines into aldehydes (Barakat and Mousa, 1952). Although this reaction seemed unlikely in view of the mild conditions of the tyrosine cleavage, it might, by placing a carbonyl dipole adjacent to the amide carbonyl, prevent its participation in the cleavage.

A third possible role for the free amino group of tyrosine might be its direct participation in a reaction route alternative to, and preclusive of, the cleavage mechanism. One such route might be a Michael-type addition (VIII) to the dienone system in intermediate III. This intramolecular addition has a precedent in the well-known conversion of 3,4-dihydroxyphenylalanine (dopa) derivatives into 5,6-dihydroxyindoles with oxidants, a reaction involving a Michael-type addition to an intermediate *o*-quinone, dopa-quinone (Sobotka *et al.*, 1957; Heacock, 1959, 1965). The work described in this paper supports the reaction mechanism of Scheme II.

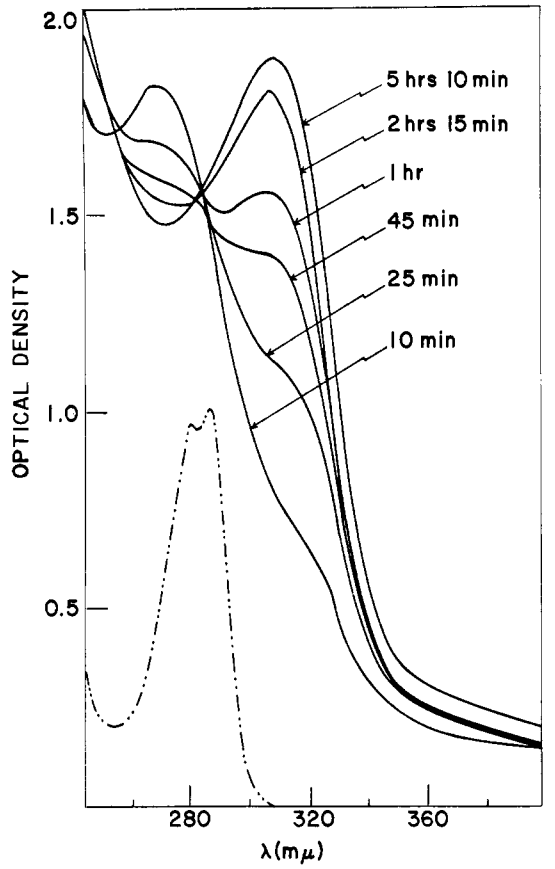


FIGURE 1: Changes in the ultraviolet spectrum of ethyl 3,5-dibromotyrosinate (—··—) on treatment with 2 equiv of NBS at room temperature in 0.25 M acetic acid.

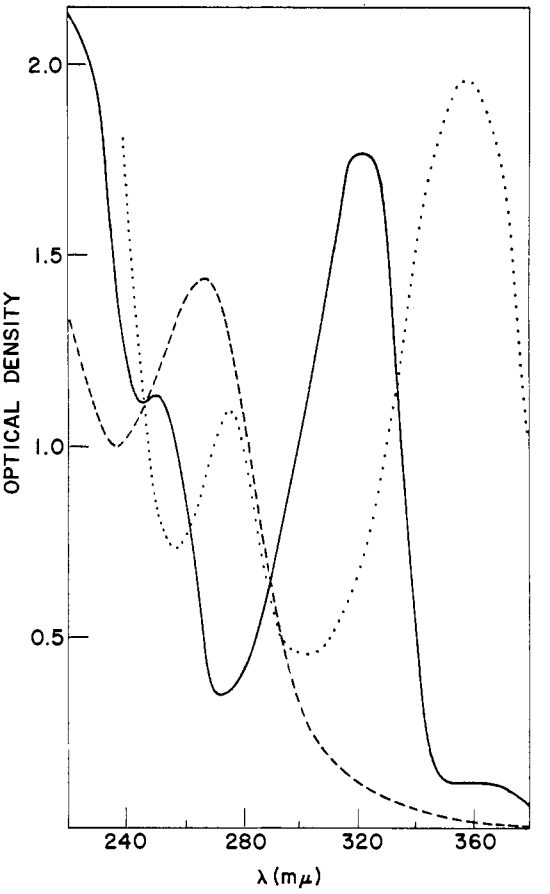


FIGURE 2: Ultraviolet spectra (ethanol) of the tribromodienone XVIII (– – –) and the indole IX (——, acid medium; · · · ·, alkaline medium) obtained on oxidation of ethyl ester with NBS. Although the concentrations are not equivalent for convenience sake, the data are presented on the same scale.

Results and Discussion

The tyrosine derivatives VIIa–f in 0.25 M aqueous acetic acid solution were treated with aqueous NBS solution and the ultraviolet spectrum was scanned after the addition of 1, 2, 3, 4, or more equiv of NBS. After the addition of 1 equiv of NBS all of these derivatives increased their absorbance at 275–280 mμ, followed by an identical increase in absorbance on addition of the 2nd equiv of NBS. This spectral change is characteristic of *ortho* bromination of the phenolic hydroxyl group. The addition of a 3rd equiv of NBS resulted in rapid and large increases in absorbance and a shift to λ_{max} 260–270 mμ. The addition of a 4th equiv did not change the spectrum appreciably, and excess NBS was present in the ultraviolet cuvet. The addition of 1 equiv of NBS to 3,5-dibromotyrosine methyl ester or 3,5-dibromotyrosine ethyl ester gave final spectra resembling the addition of 3 equiv of NBS to VIIa or b. 3,5-Dibromotyrosines are intermediates in the treatment of VIIa or b with 3 equiv of NBS.

The absorption λ_{max} 260–270 mμ of the unstable intermediate, resulting from the reaction of 3 equiv of NBS on derivatives VIIa–f, has approximately the same extinction as the spirodienonelactone V (Schmir *et al.*, 1959). However, this new compound VIII differs significantly from V in the following three respects: (i) the spectrum disappeared instantaneously on treatment with thiosulfate; (ii) the spectrum decreased steadily on standing or disappeared rapidly on warming for several minutes on the steam bath; (iii) commensurate with the decrease of absorption of λ_{max} 260–270 mμ a new absorption at 315 mμ appeared characteristic of the indole IX (Figure 1). These properties contrast with those of the spirodienonelactone V which is stable to the action of thiosulfate and which has to be refluxed with 4.0 N sulfuric acid for rearrangement to a resorcinol (Schmir *et al.*, 1959).

The 315-mμ absorbance reached a maximum value either on standing at 25° for about 16 hr, or on warming on a steam bath for 5–10 min after λ_{max} 260–270 mμ had reached its maximum intensity. The 315-mμ absorption was further increased by the addition of a 4th equiv of NBS. The final molar extinction coefficient at 315 mμ reached values of 4300–5000 which corresponds to 25% of the extinction of IX (ε 18,000).

When tyrosine, 3,5-dibromotyrosine, and tyramine were oxidized with NBS in the same fashion, no products with absorption at 315 mμ were observed.

In order to identify the two NBS conversion products with λ_{max} 270 and 315 mμ (Figure 2), respectively,

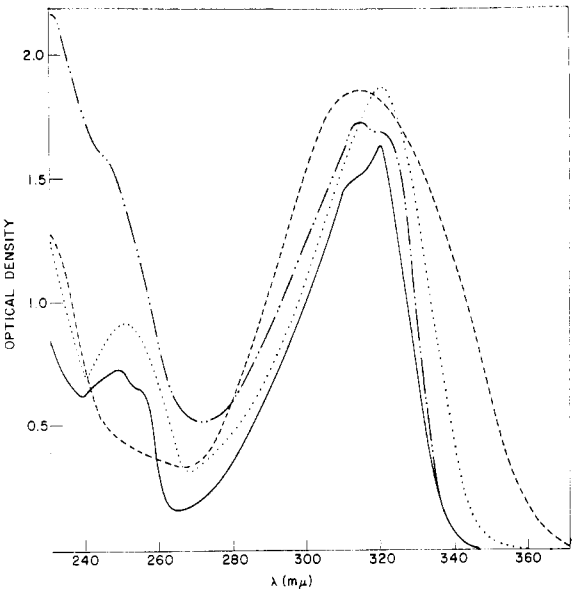
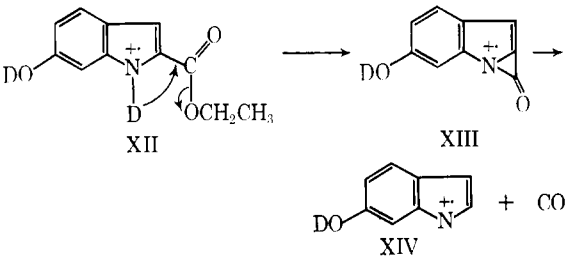


FIGURE 3: Ultraviolet spectra (ethanol) of representative 6-hydroxy- and 5,6-dihydroxyindoles: (X) c 0.94 × 10^{-4} M (· · · ·), (XI) c 0.86 × 10^{-4} M (——), (XV) c 1.05 × 10^{-4} M (—· ·—), and (XXV) c 1.05 × 10^{-4} M (– – – –).

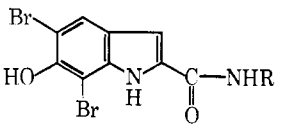
tyrosine ethyl ester was allowed to react with 3 and 4 equiv of NBS in 40% aqueous acetic acid on a preparative scale. After reaction with 3 equiv of NBS the mixture was extracted with ether. After purification the tribromodienone XVIII was isolated in crystalline form. The sharp infrared absorption band at 1600 cm^{-1} is typical of the double-bond stretching vibration of dienones. The dienone XVIII on standing in ethyl acetate–ether solution reverted to and deposited ethyl 3,5-dibromotyrosinate (XVII), concomitant with the formation of the indole IX. This indole was obtained in a more direct way by treating ethyl tyrosinate with 4 equiv of NBS. When all of the NBS had been consumed, the reaction mixture was extracted with ethyl acetate and the extract was chromatographed on a column of silica gel with chloroform–methanol (9:1). The main fraction (20–25% yield) was the indole which was catalytically debrominated to X and then methylated to give the known ethyl 6-methoxyindole-2-carboxylate (XI). The melting point and ultraviolet absorption spectrum (λ_{max}^{EtOH} 315 mμ (ε 20,800)) agree well with the data reported in the literature (Pappalardo and Vitali, 1958) (Figure 3).

A low-resolution mass spectrum (Figure 4A) of XI exhibited a parent molecular ion at m/e 219 and fragments consistent with the loss of CH_3, C_2H_5OH, C_2H_5O, C_2H_4, CO (twice), and CN. Two fairly strong metastable peaks were observed (121.7 and 134.6 m/e units) consistent with the consecutive loss of C_2H_5-OH and CO. A high-resolution mass spectrum of ethyl 6-hydroxyindole-2-carboxylate (X) (Figure 4B) had a molecular ion ($M\cdot^+$) at m/e 205.073 (calcd for $C_{11}H_{11}NO_3$: 205.074) and principal peaks at 159.033 (calcd for $C_9H_5NO_2$: 159.032 ($M\cdot^+ - C_2H_5OH$)); 131.037 (calcd for C_8H_5NO: 131.037 ($M\cdot^+ - C_2H_5OHCO$)) and 101.026 (calcd for C_7H_3N: 101.027 ($M\cdot^+ - C_2H_5OH-2CO-2H$)).

A low-resolution mass spectrum of X, after deuteration, revealed a series of molecular ions (in order of decreasing intensity) at 207, 208, and 209 corresponding to the introduction of 2, 3, and 4 atoms of deuterium into XIII. The loss of C_2H_5OD from the parent (207) suggests the unusual fragmentation pathway XII–XIII–XIV.



The oxidation of tyrosineamide (VIIc) and *N*-methylamide (VIId) by NBS paralleled the results observed with the methyl and ethyl esters. The dibromoindole derivatives XV and XVI were obtained crystalline in 20% yield after chromatography. A high-resolution mass spectrum of XV gave a triplet of peaks



XV, R = H
XVI, R = Me

for the molecular ions with an intensity ratio of 1:2:1 at m/e 331.879, 333.874, and 335.873 consistent with the formula $C_9H_6Br_2N_2O_2$ (calcd for ^{79}Br–^{79}Br: 331.880; calcd for ^{79}Br–^{81}Br: 333.875; calcd for ^{81}Br–^{81}Br: 335.878). Another such triplet at m/e 314.989, 316.853, and 318.985 was observed corresponding to $C_9H_3Br_2NO_2$ (calcd: 314.983, 316.851, and 318.979 for the permutations of ^{79}Br and ^{81}Br) represented a loss of NH_3 from the parent. Additional fragments observed as principal peaks in the mass spectrum were found consistent with $C_8H_5Br_2NO$ ($M\cdot^+ - HNCO$), C_8H_3Br-NO ($M\cdot^+ - NH_3$, – CO, and – Br), C_8H_4BrNO ($M\cdot^+$ – NH_2, – CO, and – Br), C_8H_6BrNO ($M\cdot^+$ – NCO and – Br), C_7H_4BrN ($M\cdot^+ - NH_2$, – 2CO, and – Br), C_8H_4NO ($M\cdot^+ - NH_2$, – CO, and – 2Br), C_8H_7NO ($M\cdot^+$ – NCO and – 2Br + H). A series of peaks from 100 to 104 were found whose exact masses corresponded to the nitrogen-containing core, $C_7H_{2-6}N$. The extrusion of CO from phenols is well documented (Budzikiewicz *et al.*, 1964) and has also been found in other phenolic heterocycles, for instance in 8-hydroxyquinolines (Brown, 1965). The nuclear magnetic resonance spectrum of XV was measured in CD_3OD for reasons of solubility. It showed a single proton at 7.2 ppm. All other protons had been exchanged for deuterium during the time required to prepare the sample. The position of this signal is typical of the proton in the 4 position of 5,6-diacetoxy-7-iodo-2-substituted indoles (Heacock *et al.*, 1963).

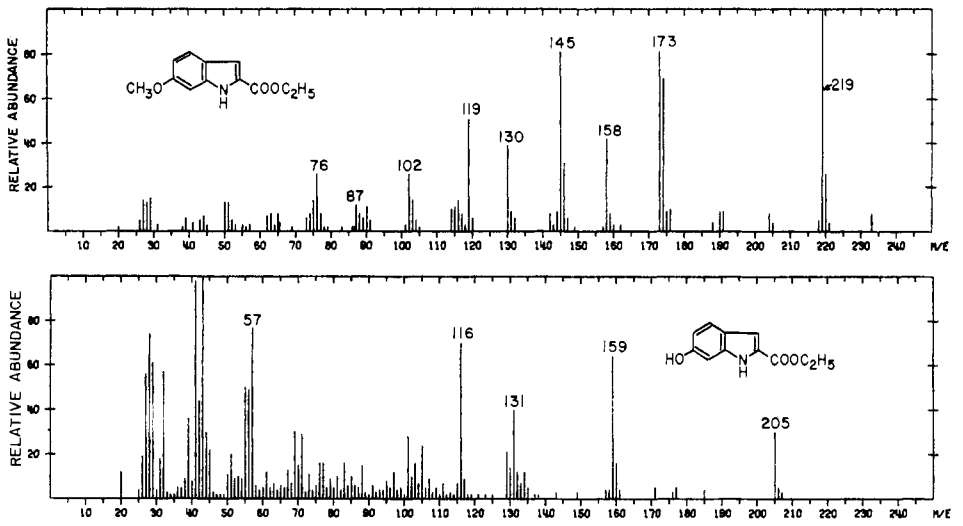


FIGURE 4: Low-resolution mass spectra. (A, top) Of ethyl 6-methoxyindole-2-carboxylate (XI) and (B, bottom) of ethyl 6-hydroxyindole-2-carboxylate (X).

In an effort to improve the yield of the indole IX, the addition of NBS to tyrosine ethyl ester was carried out first under acidic conditions as before, and then 30–60 min after the consumption of NBS was complete the pH was raised to approximately 6 by the addition of bicarbonate. Unexpectedly, the main product, isolated in 20% yield, was not the dibromohydroxyindole IX, but rather a dihydroxybromoindole which was shown to be ethyl 5,6-dihydroxy-7-bromoindole-2-carboxylate (XXV) by comparison of this material and its diacetate XXXIII with the analogous compounds prepared by NBS oxidation of 3,4-dihydroxyphenylalanine (dopa) ethyl ester.

The high-resolution spectrum showed the parent mass of the dihydroxyindole XXV as a doublet of *m/e* 298.979 and 300.979 consistent with the formula $C_{11}H_{10}BrNO_4$ (calcd for ^{79}Br and ^{81}Br: 298.979 and 300.977, respectively). Low-resolution mass spectra of XXV obtained either from XVII or dopa were identical. The reaction of dopa ethyl ester with NBS is comparable to oxidation with potassium iodate which leads to the analogous ethyl 5,6-dihydroxy-7-iodoindole-2-carboxylate (Heacock *et al.*, 1963).

The conversion of XVII into the dihydroxyindole XXV at pH 6 involves displacement of one nuclear bromine substituent by hydroxyl. This loss of bromine which occurs in the presence of excess NBS is unusual. While a large number of derivatives of 3,4-dihydroxyphenylethylamine have been converted into 5,6-dihydroxyindole derivatives using mild oxidants such as ferricyanide or silver oxide (*e.g.*, Bu'Lock and Harley-Mason, 1951; for a review, see Heacock, 1965), we are unaware of any instance of a strictly chemical conversion of monohydroxylated starting material (*e.g.*, tyrosine ester) into a 5,6-dihydroxyindole.

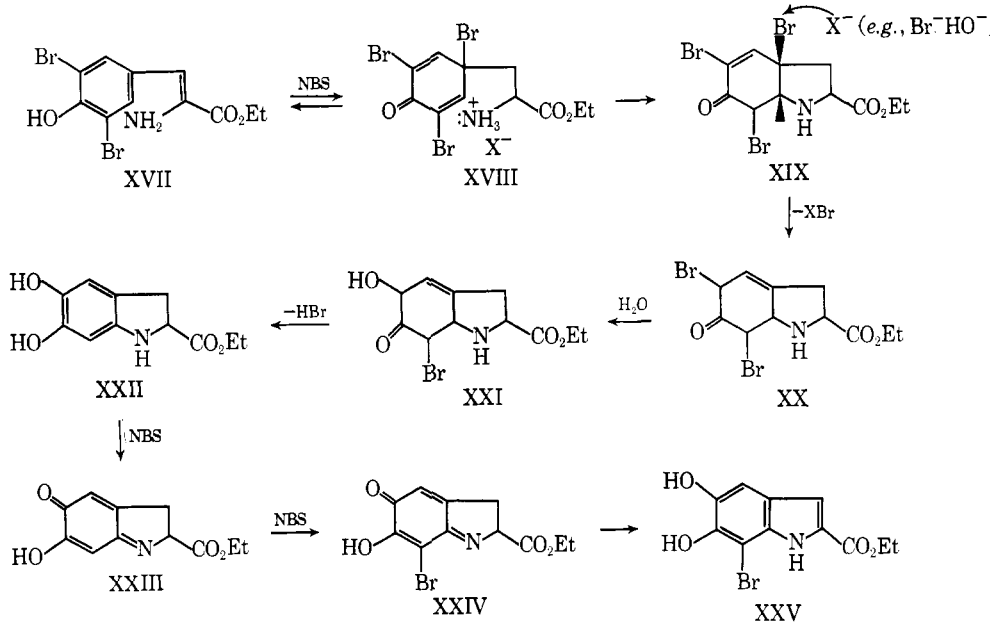
A colored by-product was isolated in low yield which exhibited a broad absorption band in the visible region, λ_{max} 480 mμ, characteristic of aminochromes. In line with this observation Scheme III, which may be arbitrary with regard to detail and sequence of events, is to a large extent analogous to the formulation of the pathway leading from dopa to aminochrome intermediates such as XXIII and XXIV. The loss of bromine from intermediate XIX might be considered analogous to the reversion of XVIII to XVII in HBr solution, a reductive debromination, presumably catalyzed by bromide ion. The stepwise oxidation of the dihydrohydroxyindole XXII to the aminochrome XXIII and conversion into the 7-bromoaminochrome XXIV is suggested by recent studies on the oxidation of adrenaline with iodine where iodination was shown to occur after the formation of adrenochrome (Mattok and Wilson, 1967). The rearrangement of the bromoaminochrome XXIV to the indole XXV parallels the sequence of steps outlined by Raper (Raper, 1927; Heacock, 1965).

The dibromohydroxyindoles (XXIX, R = OMe, OEt, NH_2, NHMe, NHCH(CH_3)COOH) obtained under acidic conditions may be formed by the sequence XXVI–XXIX in Scheme IV. The *cis*-ring junction in the tribromoenone XXVI would be the preferred configuration of a bicyclic 5,6 system. In order to avoid *cis* elimination a two-step loss (E-1 type, XXVII) of the elements of HBr could lead to the dihydroindole XXVIII which would be expected to undergo facile dehydrogenation to the indole XXIX.

A 2,3-dihydroindole XXXI (Scheme V) was isolated in the NBS oxidation of dopa ester III, when the intermediate aminochrome was reduced by dithionite. After acetylation, ethyl 5,6-diacetoxy-7-bromoindole-2-carboxylate (XXXII) was isolated as the major product. The 2,3-dihydroindole XXXII represented approximately 10% of the reaction product and was characterized and identified by nuclear magnetic resonance, infrared, ultraviolet, and mass spectra.

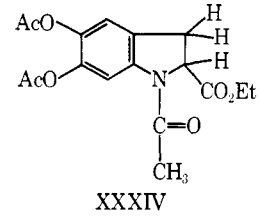
On standing for several minutes in an alkaline alcoholic solution, its ultraviolet spectrum was identical with that of the indole XXV. The dehydrogenation of the 2,3-dihydroindole XXXII to the indole XXV under alkaline conditions is a spontaneous process. The

SCHEME III



nuclear magnetic resonance spectrum of XXXII (see Experimental Section) is very similar to that reported for DL-indoline-2-carboxylic acid (Hudson and Robertson, 1967).

A third material was isolated in low yield and impure condition from the NBS oxidation of dopa ester XXX. Its mass, infrared, ultraviolet, and nuclear magnetic resonance spectra are consistent with its formulation as the bromine-free, *O,O,N*-triacetate XXXIV.



XXXIV

This material displayed a blue fluorescence when excited with ultraviolet light. Apparently the bulky bromine atom in the 7 position, adjacent to the indoline nitrogen, prevents acetylation in the case of XXXII under the mild acetylation conditions employed. The occurrence of this material could result either from reduction of unbrominated dopachrome or from a reductive dehalogenation of a bromodopachrome by dithionite. Iodine is sometimes lost from iodoaminochromes on reduction with dithionite or similar reducing agents. The formation of these 2,3-dihydroindoles (leucoaminochromes) in the oxidation of catecholamines seems to be relatively rare, even under conditions where the intermediate aminochromes are reduced before isolation of the reaction products. Ethyl 2,3-dihydro-5,6-diacetoxy-7-iodoindole-2-carboxylate has been prepared by reduction of 7-iododopachrome ethyl ester with dithionite (Bu'Lock and Harley-Mason, 1951).

The oxidation of tyrosyl residues to 6-hydroxyindoles offers a convenient method for the rapid determination of NH_2-terminal tyrosine in peptides, proteins, and protein digests. This method will be described in the subsequent paper (Wilchek *et al.*, 1967).

Experimental Section

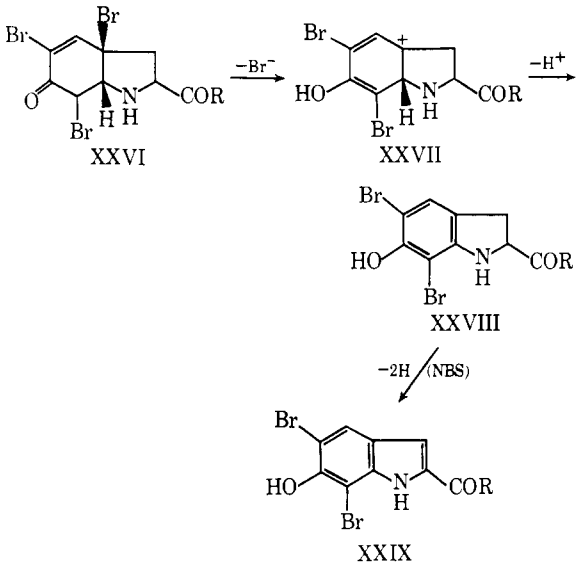
Melting points were determined on a Köfler micro hot stage and are uncorrected. Mallinckrodt silicic acid was used for chromatography. Vapor phase chromatography used a Glowall Model 310 with an OV-17 (3%) column packing. Column temperatures are given in parentheses.

*3,5-Dibromo-*L*-tyrosine Ethyl Ester Hydrochloride (XXXIV).* 3,5-Dibromo-L-tyrosine (6.8 g) was added to a cold solution of thionyl chloride (3 ml) in anhydrous ethanol (100 ml). The reaction mixture was allowed to stand for 12 hr at room temperature and then concentrated until crystals began to appear. Dry ether was then added and the resulting crystals were collected by filtration. The ester was chromatographically pure (thin-layer chromatography slide, silica gel G, 1-butanol–acetic acid–water (25:6:25)): yield, 95%; mp 116°; lit. (Abderhalden and Mahn, 1928) mp 115–116°.

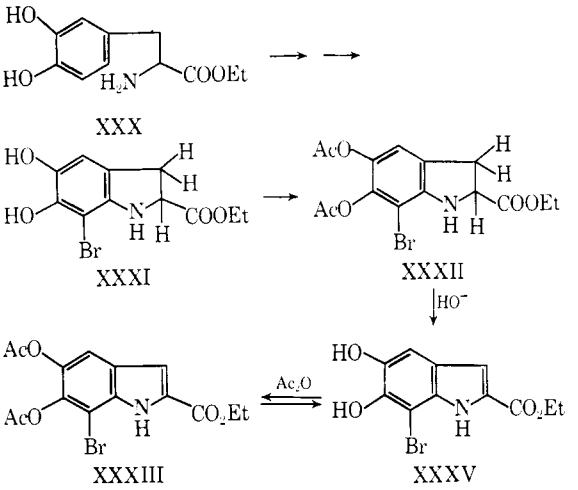
*N-Carbobenzyloxy-*L*-tyrosineamide.* To a solution of 15.7 g of carbobenzyloxy-L-tyrosine (50 mmoles) was added at 0–5°, 5.8 g of ethyl chloroformate (55 mmoles). After 10 min, 50 ml of concentrated ammonium hydroxide solution was added and the reaction mixture was allowed to stand for 12 hr. Water was then added and the crystalline precipitate was collected and washed with water, 1.0 N hydrochloric acid, and again with water. The product was recrystallized from methanol. The colorless crystals (yield 13 g, 82%) had mp 151–152°.

Anal. Calcd for $C_{17}H_{18}N_2O_4$: C, 64.95; H, 5.77; N, 8.91. Found: C, 64,99; H, 5.83; N, 8.80.

SCHEME IV



SCHEME V



N-Carbobenzyloxy-L-tyrosine-N-methylamide. This material was prepared by the same method as *N*-carbobenzyloxy-L-tyrosineamide using an aqueous solution of methylamine. Recrystallization from methanol gave colorless crystals (yield 70%), mp 148–149°.

Anal. Calcd for $C_{18}H_{20}N_2O_4$: C, 65,84; H, 6.14; N, 8.53. Found: C, 65.53; H, 6.36; N, 8.41.

L-Tyrosine-N-methylamide Hydrochloride (VIId). A solution of *N*-carbobenzyloxy-L-tyrosine-*N*-methylamide was hydrogenated in methanol containing 1 equiv of 1.0 N hydrochloric acid in the presence of 0.5 g of 10% palladium on charcoal. After 2 hr the catalyst was removed by filtration. The filtrate was evaporated *in vacuo* and the product recrystallized from methanol–ether to give colorless crystals (yield 78%), mp 235–240°.

Anal. Calcd for $C_{10}H_{14}N_2O_2 \cdot HCl$: C, 52.17; H, 6.52; Cl, 15.43; N, 12.17. Found: C, 52.18; H, 6.41; Cl, 15.42; N, 12.12.

L-Tyrosineamide (VIIc). This compound was prepared by catalytic debenzylation of *N*-carbobenzyloxy-L-tyrosineamide in acetic acid. The colorless crystals (yield 72%) had mp 176° (lit. (Behrens and Bergmann, 1939) mp 177°).

Tribromodienone Hydrobromide (VIIIf) from L-Tyrosyl-L-alanine (VIIf). A solution of 252 mg (1 mmole) of L-tyrosyl-L-alanine in 10 ml of 30% aqueous acetic acid was oxidized with 534 mg (3 mmoles) of NBS. After 10 min crystals deposited which were collected after 1 hr and washed with water. The colorless crystals (yield 92%) had mp 133–136° dec, λ_{max}^{EtOH} 270 mμ (ε 6000).

Anal. Calcd for $C_{12}H_{13}Br_3N_2O_4 \cdot HBr \cdot H_2O$: C, 24.52; H, 2.72; N, 4.76. Found: C, 24.22; H, 2.71; N, 5.45.

Tribromodienone Hydrochloride (XVIII) of L-Tyrosine Ethyl Ester (VIIb) Hydrochloride. A solution of 245 mg of L-tyrosine ethyl ester hydrochloride in 10 ml of 40% aqueous acetic acid was oxidized with 534 mg of NBS. After 20 min water was added and the reaction mixture was extracted with ether. The ether solution was dried over sodium sulfate and evaporated to dryness. The residue was chromatographed on a column of silica gel (30 g). Elution with 6% methanol in chloroform gave 200 mg (41%) of tribromodienone hydrochloride (XXI), mp 101° dec, λ_{max}^{EtOH} 270 mμ.

Anal. Calcd for $C_{11}H_{12}Br_3NO_3 \cdot HCl$: C, 27.38; H, 2.69; N, 2.90; Hal, 57.06. Found: C, 27.44; H, 2.41; N, 2.90; Hal, 58.24.

Reversion to Dibromotyrosine Ethyl Ester. When the dienone (XVIII) was dissolved in ethyl acetate and ether added and the solution left for 2 days, the crystalline compound which precipitated was identified as 3,5-dibromotyrosine ethyl ester hydrobromide.

Ethyl 5,7-Dibromo-6-hydroxyindole-2-carboxylate (IX). A solution of 245 mg (1 mmole) of tyrosine ethyl ester hydrochloride was dissolved in 40% aqueous acetic acid and oxidized with 712 mg (4 mmoles) of NBS. The reaction mixture was left for 24 hr after which time it became black due to the formation of dark melanine-like material. The reaction mixture was extracted with ethyl acetate and the extract was evaporated to dryness. Thin-layer chromatography revealed the presence of at least six components. This mixture was chromatographed on a column of silica gel (30 g). Elution with 5% methanol in chloroform yielded 76 mg (21%) of XII as colorless crystals, mp 149–162° dec.

Anal. Calcd for $C_{11}H_9NO_3Br_2$: C, 36.36; H, 2.47; N, 3.85. Found: C, 36.5; H, 2.78; N, 4.01.

The infrared spectrum showed 3540, 3450 sharp, 1710, 1630, 1610, 1530, 1090, and 1020 cm^{-1}. The same ethyl 5,7-dibromo-6-hydroxyindole-2-carboxylate (IX) was obtained starting with 3,5-dibromo-L-tyrosine (XVII) in 20% yield.

5,6-Dihydroxy-7-bromo-2-carbethoxyindole (XXV). Subsequent elution gave a minor component (5%) which was identified as 5,6-dihydroxy-7-bromo-2-carbethoxyindole (XXV), mp 150–151° (see below).

Anal. Calcd for $C_{11}H_{10}BrNO_4$: C, 44.02; H, 3.33; N, 4.67. Found: C, 43.55; H, 3.27; N, 4.65.

Elution of the column with pure methanol gave three

additional colored fractions which looked like melanine polymers of differing molecular weights.

Ethyl 5,7-Dibromo-6-acetoxyindole-2-carboxylate. An aliquot from the reaction mixture of the preparation of IX containing 0.5 mmole of IX was evaporated *in vacuo* and 5 ml each of acetic anhydride and pyridine was added to the residue. After standing at 20° for 24 hr the solvents were removed, the residue was dissolved in ethyl acetate, and a small amount of black insoluble material was removed by filtration. Gas–liquid partition chromatography revealed the presence of two major components which on thin-layer chromatography (1% methanol–chloroform) showed R_F 0.52 and 0.29, both reacting with *p*-dimethylaminocinnamaldehyde (modified Ehrlich reagent). Chromatography on silica (30 g) with 1% methanol–chloroform (4-ml cuts) afforded 5,7-dibromo-6-acetoxyindole-2-carboxylate in the first fraction: vapor phase chromatography, homogeneous (210°), ultraviolet spectrum, 235 mμ and 295 mμ; after standing overnight with alkali, 355 mμ and 270 mμ; on reacidification, 315 mμ and 245 mμ (sh). The infrared spectrum showed 340 sharp, 1770 s, 1710 s, 1560, 1530, 1460, 1420, 1370 s, 1020, and 875 cm^{-1}; the mass spectrum, triplet at 403, 405, and 407.

Ethyl 6-Hydroxyindole-2-carboxylate (X). A solution of 100 mg of the dibromoindole XII in 80% aqueous methanol containing sodium acetate was hydrogenolyzed in a Parr apparatus for 30 min in the presence of paladium on charcoal. The reaction mixture was concentrated, dissolved in water, and extracted with peroxide-free ether. The ether extract was concentrated to dryness and the residue recrystallized from ether–petroleum ether (bp 30–60°) to give colorless crystals (yield 48 mg, 83%): mp 169–175°; $\lambda\lambda_{max}^{EtOH}$ 320 (log ε 4.32), 250 (3.95), and 215 mμ (4.28). The compound was homogeneous by the criterion of vapor phase chromatography (after formation of trimethylsilyl derivative) (240°).

Anal. Calcd for $C_{11}H_{11}NO_3$: C, 64.40; H, 5.36; N, 6.84. Found: C, 62.72; H, 5.29; N, 6.83.

The infrared spectrum ($CHCl_3$) showed 3600 (sharp), 3500 (sharp), 3460 (sharp), 1700, 1630, 1590 (sh), 1540, 1500, 1270, 1160, 960, 890, and 860 cm^{-1}.

Ethyl 6-Methoxyindole-2-carboxylate (XI). To a solution of 40 mg of ethyl 6-hydroxyindole-2-carboxylate (X) in dry acetone (10 ml) was added anhydrous potassium carbonate followed by dimethyl sulfate (1 ml). The reaction mixture was heated under reflux for 16 hr, then water (0.5 ml) was added, and refluxing was continued for an additional 10 min. The acetone was decanted and concentrated *in vacuo*. The residue was suspended in a dilute sodium bicarbonate solution, allowed to stand for 1 hr, and then extracted with ether. The ether extract was evaporated, and the residue was dissolved in ethyl alcohol and recrystallized from ethanol–water to yield colorless crystals (20 mg, 46%): mp 132–135°, lit. (Pappalardo and Vitali, 1958) mp 134–135°. The product was homogeneous by vapor phase chromatography: λ_{max}^{EtOH} 320 (log ε 4.28), 250 (3.93), 215 (4.28), and 265 mμ (3.29); infrared spectrum ($CHCl_3$), 3480 (sharp), 2940, 2850, 1700, 1630 s, 1580 w, 1530, 1470, 1430 w, 1250, 1180, 1160, and 1120 cm^{-1}.

5,7-Dibromo-6-hydroxyindole-2-carboxamide (XV). A solution of 180 mg (1 mmole) of L-tyrosineamide in 40% acetic acid was oxidized with 712 mg (4 mmoles) of NBS as described above. The reaction mixture was chromatographed on a column of silica gel (30 g) and eluted with 8% methanol in chloroform to give colorless crystals (77 mg, 23%), mp 247° dec.

Anal. Calcd for $C_9H_6Br_2N_2O_2 \cdot 0.5H_2O$: C, 31.52; H, 2.05; Br, 46.61; N, 8.14. Found: C, 31.21; H, 1.94; Br, 46.38; N, 8.07.

Other results are: $\lambda\lambda_{max}$ 320 (ε 4.21), 313 (4.22), 245 (sh) (4.18), and 227 mμ (4.33) in 95% EtOH containing mineral acid; $\lambda\lambda_{max}$ 355 mμ (ε 4.32) and 273 mμ (ε 4.04) in alkaline 95% EtOH.

5,7-Dibromo-6-hydroxyindole-2(N-methylcarboxamide) (XIV). A solution of 230 mg of L-tyrosine *N*-methylamide (1 mmole) in 30% acetic acid was oxidized with 712 mg of NBS (4 mmoles). The mixture which according to thin-layer chromatography contained at least six products was chromatographed on silica gel and eluted with 7% methanol in chloroform. The yield was 60 mg (17%). This compound was recrystallized from ethyl acetate–ether: mp 172–175°, λ_{max}^{EtOH} 315 mμ.

Anal. Calcd for $C_{10}H_8Br_2N_2O_2$: C, 34.48; H, 2.29; Br, 45.98; N, 8.04. Found: C, 34.21; H, 2.54; Br, 46.20; N, 7.73.

Ethyl 5,6-Dihydroxy-7-bromoindole-2-carboxylate (XXV). A. FROM ETHYL L-TYROSINATE. To a solution of 245 mg (1 mmole) of L-tyrosine ethyl ester hydrochloride in 20 ml of 20% acetic acid was added 712 mg (4 mmoles) of NBS. After 10 min 2.0 N NaOH was added until the pH reached 7. The reaction mixture at this point was black. After 30 min it was acidified and extracted with ether. The ether extract was washed with water and dried over sodium sulfate. Thin-layer chromatography revealed several spots. This mixture was chromatographed on silica gel (30 g). Elution with 5% methanol in chloroform yielded 84 mg (28%) of colorless crystals: mp 152–155°; $\lambda\lambda_{max}$ 315 (ε 4.25) and 227 mμ (ε 4.15) in 95% EtOH containing HCl; $\lambda\lambda_{max}$ 357 (ε 4.33), 265 (3.75), and 223 mμ (4.17) in alkaline 95% EtOH; vapor phase chromatography, less than 1% impurity (on the trimethylsilyl derivative) (240°). This compound had the same retention time as the analogous material isolated from dopa ester.

Anal. Calcd for $C_{11}H_{10}BrNO_4$: C, 44.02; H, 3.33; Br, 26.63; N, 4.67. Found: C, 43.75; H, 3.35; Br, 26.69; N, 4.91.

The infrared spectrum ($CHCl_3$) showed 3570 (sharp), 3460 (sharp), 3215 (broad), 1700, 1640 w, 1570 w, 1540, 1510, 1460, 1420, 1310 (sharp), 1260, 1020, and 850 cm^{-1}. A minor component (5%) was eluted and identified as ethyl 5,7-dibromo-6-hydroxyindole-2-carboxylate (IX).

Elution of the column with ethyl acetate gave a deep red unstable aminochrome (3%; λ_{max} 480 mμ and 320 mμ) which on concentration turned black. Further elution with pure methanol gave three additional brown fractions which had the characteristic of melanin-like products.

B. FROM 3,5-DIBROMO-L-TYROSINE ETHYL ESTER (XVII). A solution of 400 mg (1 mmole) of 3,5-dibromotyrosine in 30 ml of 20% acetic acid was oxidized with 356 mg of NBS (2 mmoles) as described above. After chromatog-

raphy on silica gel 78 mg (26%) of colorless crystals was obtained, mp 152–155°. Ultraviolet, infrared, and elemental analyses and mobility on thin-layer chromatography were identical with ethyl 5,6-dihydroxy-7-carboxylate (XXV). The other minor components were also present in the reaction mixture.

C. FROM 3,4-DIHYDROXYPHENYLALANINE ETHYL ESTER (DOPA ETHYL ESTER, XXX). To a solution of 261 mg (1 mmole) of 3,4-dihydroxyphenylalanine ethyl ester hydrochloride in 20% acetic acid (12 ml) was added a solution of 534 mg (3 mmoles) of NBS in 20 ml of the same solvent. The solution became immediately red and smelled of bromine. After a few minutes the color of the solution was discharged to yellow and slowly returned to red (λ_{max} 460–520 mμ). On standing for 16 hr at room temperature the solution turned black. This solution was extracted with ether, and the ether extract was dried over sodium sulfate and evaporated to dryness. The residue contained five spots on thin-layer chromatography. The mixture was chromatographed on silica gel (30 g) and eluted with 5% methanol in chloroform. The yield was 78 mg (25%), mp 149–153. This compound was identical with ethyl 5,6-dihydroxy-7-bromoindole-2-carboxylate prepared from tyrosine ethyl ester by the criteria of mass spectra, ultraviolet, and vapor phase chromatography retention time (<4%) impurity ("OV-17" (240°). An impurity (3%) of exactly twice the retention time was measured on OV-17 (225°) on trimethylsilyl derivative.

5,6-Dihydroxy-7-bromoindole-2-carboxamide. A solution of 180 mg (1 mmole) of L-tyrosineamide in 20% acetic acid was oxidized with 712 mg (4 mmoles) of NBS. After 10 min the pH was adjusted to 6–7 by the addition of 2.0 N NaOH. Acidification, extraction, followed by silica gel chromatography (8% methanol in chloroform) yielded 73 mg (27%) of colorless crystals, mp 210–220° (dec), λ_{max}^{EtOH} 315 mμ.

Anal. Calcd for $C_9H_7BrN_2O_3$: C, 40.00; H, 2.59; N, 10.33. Found; C, 40.12; H, 2.62; N, 10.31.

A *minor component* (6%) with very similar chromatographic properties was also isolated. The analysis of this compound, which is significantly higher in hydrogen is consistent with $C_9H_9BrN_2O_3$: *i.e*, 2,3-dihydroindole-2-carboxamide.

Anal. Calcd for $C_9H_9BrN_2O_3$: C, 39.57; H, 3.29. Found: C, 39.64; H, 3.32.

This material which was not further characterized corresponds to the 2,3-dihydroindole isolated from the NBS oxidation of dopa ethyl ester. In addition to these two products there was also isolated in low yield 5,7-dibromo-6-hydroxyindole-2-carboxamide.

Ethyl 5,6-Diacetoxy-7-bromoindole-2-carboxylate (XXXIII) and Ethyl 5,6-Diacetoxy-7-bromo-2,3-dihydroindole-2-carboxylate (XXXII) (from Dopa Ethyl Ester). A solution of 523 mg (2.00 mmole) of dopa ethyl ester hydrochloride (lit. (Bu' Lock and Harley-Mason, 1951) mp 98–105°) in 50 ml of 0.1 M acetate buffer (pH 5.0) was oxidized with stirring at 20° with 723 mg (4.06 mmoles) of NBS and stirred for 2 hr until starch–KI paper was no longer blue. The solution initially turned deep orange and the odor of bromine was noted. After 2 hr the solution was deep red in color with no free bromine present. A second portion of 368 mg (2.07 mmoles) of NBS was added with rapid stirring. The solution which had become brown-red and slightly turbid was stirred for 3 hr until free bromine had disappeared. An excess of solid sodium dithionite was then added. The resulting brownish suspension was extracted with two portions of ethyl acetate and the extract was washed with water until neutral and then dried (Na_2SO_4). After evaporation of the ethyl acetate, the oily residue was acetylated in 10 ml of acetic anhydride with 10 ml of pyridine added. The acetylation mixture was allowed to stand overnight at room temperature, then freed of solvents (35°, *in vacuo*). The oily residue was chromatographed on a column (16 × 315 mm) of silica gel. Two main fractions were obtained (4-ml cuts, 1% methanol–chloroform as eluent): (A) 146 mg of colorless oil (19% yield, calculated as mol wt 384) positive test with modified Ehrlich reagent, positive Beilstein test for bromine; (B) 71 mg of red-brown oil, vapor phase chromatography reveals at least seven components, though one comprises 80% of the fraction; positive Ehrlich and Beilstein tests.

Fraction A was separated into two components by fractional crystallization from ethyl acetate–hexane: a high-melting indole fraction, needles, mp 197–198°; and a lower melting dihydroindole, yellowish prisms, mp 128–130°. The compounds had the same R_F and gave a deep red-purple color with modified Ehrlich spray, although after an initial exposure to iodine vapor only the lower melting product gave a positive test. These products were identified as ethyl 5,6-diacetoxy-7-bromoindole-2-carboxylate, mp 198° (XXXIII), and the corresponding 2,3-dihydroindole (XXXII).

COMPOUND XXXIII. Nuclear magnetic resonance (CD_3CN) showed 1.39 (triplet, J = 7 cps, three protons, $CO_2CH_2CH_3$), 2.30, 2.37 (singlets, each three protons, O*Ac*), 4.42 (quartet, J = 7 cps, two protons, $CO_2CH_2CH_3$), 7.32 (singlet, one proton, 4H), and 7.53 ppm (singlet, one proton, exchanges with D_2O, NH); infrared spectrum ($CHCl_3$) 3450 (sharp), 1770 s, 1710 s, and 1370 s cm^{-1}; ultraviolet spectrum (EtOH) λλ$_{max}$ 230 mμ and 295 mμ; absorption shifts to 360 mμ immediately on treatment with a few drops of 1 N NaOH, shifts to 315 mμ on reacidification; mass spectroscopy $M\cdot^+$ doublet at 383, 385; loss of CH_2CO (twice), EtOH, CO (twice).

Anal. Calcd for $C_{15}H_{14}BrNO_6$: C, H, 46.90; H, 3.65; Br, 20.85. Found: C, 46.60; H, 3.47; Br, 21.19.

DIHYDROINDOLE XXXII. Nuclear magnetic resonance ($CDCl_3$) showed (triplet, J = 7 cps, three protons, $CO_2CH_2CH_3$), 2.31, 2.39 (singlets, three protons each, O*Ac*), 3.55 (doublet, J = 8 cps, two protons, 3 position), 4.37 (quartet, J = 7 cps, two protons, $CO_2CH_2CH_3$), 4.62 (triplet, J = 8 cps, one proton, 2 position), 7.07 (singlet, one proton), and 7.47 ppm (broad, *ca.* one proton, NH ?); infrared spectrum ($CHCl_3$) 3500 w (broad), 1770, 1740, 1620, 1590, 1470 s (sharp), and 1370 cm^{-1}; ultraviolet spectrum (EtOH), 303 mμ, after OH^- (360 mμ, maximum optical density reached in *ca.* 1 min), on reacidification λ_{max} shifts to 315 mμ; mass

spectroscopy $M \cdot^+$ doublet at 385, 387.

Anal. Calcd for $C_{15}H_{16}BrNO_6$: C, 46.60; H, 4.15; Br, 20.70; N, 3.63. Found: C, 45.20; H, 4.31; Br, 21.15; N, 3.72.

Ethyl N-Acetyl-5,6-diacetoxy-2,3-dihydroindole-2-carboxylate (XXXIV). To 2.01 mmoles of dopa ester hydrochloride in 50 ml of 0.1 N HOAc (pH 3.2) was added, with rapid stirring, 4.11 mmoles of NBS dissolved in 50 ml of 0.1 N HOAc. After 1.5 hr (no free bromine present), 2.09 mmoles of NBS in 25 ml of 0.1 N HOAc was added and the reaction mixture was stirred for 5 hr at room temperature. After reduction with excess sodium dithionite, the reaction mixture was worked up as above to yield an oily residue, which was acetylated (5 ml each acetic anhydride and pyridine, 2 hr, room temperature) to afford 296 mg of acetylated product. After chromatography under the same conditions as above, 157 mg (20.4%) of a colorless oil was obtained (fraction A) which was shown by vapor phase chromatography to consist of a 2:3 mixture of the dihydroindole XXXII and the indole XXXIII, respectively. From the three cuts (total volume 12 ml) immediately following this fraction, there was isolated, after repeated crystallization from ethyl acetate–hexane, colorless crystals (mp 127–130°), which exhibited a blue fluorescence on thin-layer chromatography. This product was identified as *ethyl N-acetyl-5,6-diacetoxy-2,3-dihydroindole-2-carboxylate (XXXII)*: infrared spectrum ($CHCl_3$) 3460 w (indole, impurity) 1770, 1710, 1670, 1530, 1490 (sharp), 1400, and 1370 cm^{-1}; nuclear magnetic resonance spectrum ($CDCl_3$) 1.26 (triplet, J = 6 cps, three protons, $CO_2CH_2CH_3$, 2.24 (singlet, nine protons, $OC(=O)CH_3$, $NC(=O)CH_3$), 3.32 (broad, two protons, 3 position), 4.22 (quartet, J = 6 cps, two protons $CO_2CH_2CH_3$), 6.92 (broad, one proton in 7 position), and 7.18 ppm (doublet, J = 1.5 cps, proton in 4 position); ultraviolet spectrum λ_{max}^{EtOH} 295 mμ; slow and complex changes in base; mass spectrum $M \cdot^+$ 349, peaks for loss of CH_2CO (three times); loss of EtOH and CO.

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