Biochemical Pharmacology, Vol. 19, pp. 1991–2000. Pergamon Press. 1970. Printed in Great Britain

INTERACTION OF SEROTONIN WITH THE CATECHOLAMINES—I

INHIBITION OF DOPAMINE AND NOREPINEPHRINE OXIDATION

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(*Received* 21 *June* 1969; *accepted* 31 *October* 1969)

Abstract—Serotonin proved to be an effective inhibitor of both the enzymatic and autoxidation of dopamine and norepinephrine. Kinetic studies of the inhibition of the enzymatic oxidation indicated that serotonin forms an inhibitor–substrate complex. Structure–activity relationships indicate that the ring hydroxyl group of serotonin and the two ring hydroxyl groups of the catecholamines are the only requirements for interaction to occur. The possibility that serotonin modulates the activities of the catecholamines on a purely molecular level through complex formation has been discussed.

ALTHOUGH much work has been done to implicate both the catecholamines and serotonin in the control of behavior, little has been resolved concerning their roles in this respect. Studies in this laboratory have centered around the hypothesis that a more intimate relationship exists between these groups of compounds than would be evident if they were simply transmitters of two opposing neuronal systems as currently believed.[1] Since VanderWende and Spoerlein[2] and VanderWende[3] suggested that intermediates in pigment formation from dopamine or dopamine-melanin (pigment formed from the oxidation of dopamine exhibiting all the properties of melanin) itself may play a role in abnormal behavior, it seemed imperative to determine whether serotonin would modify the oxidation of the catecholamines and therefore exert a control over this process. From our studies, it became apparent that serotonin is an effective inhibitor of pigment formation from dopamine not only by brain preparations and purified tyrosinase but also from its autoxidation.

Inhibition of dopamine oxidation in the absence of enzyme protein led us to examine whether serotonin could complex the dopamine. It was believed that demonstration of complex formation between these two compounds could provide the basis for a hypothesis that serotonin acts to modulate the activity of dopamine (catecholamines) on a purely molecular level.

This report presents the experimental work on inhibition of pigment formation from dopamine and norepinephrine by serotonin, the mechanism of which appears to be inhibitor–substrate complex formation. The effect of serotonin on epinephrine oxidation is reported in the following companion paper.[4]

MATERIALS AND METHODS

Male albino rats obtained from K-G Farms were used as the source for brain tissue.

Mushroom tyrosinase, dopamine HCl, serotonin creatinine sulfate, 5-hydroxy indole-3-acetic acid (monodicyclohexylammonium salt), 5-methoxy-typtamine, tryptamine HCl, indole-3-acetic acid, indole, tyramine HCl, and tyrosine HCl were purchased from Sigma Chemical Co. 3-Methoxy dopamine, 4-methoxy dopamine and 3,4 dimethoxy dopamine were obtained from Calbiochem. Bufotenin bioxalate, epinephrine HCl, and phenylethylamine were purchased from Mann Research Labs.; melatonin and norepinephrine HCl from Nutritional Biochemicals Corp.; and 3,4-dihydroxyphenylacetic acid from Aldrich Chemical Co.

Dopamine and norepinephrine oxidation

Pigment formation from dopamine catalyzed by brain preparations was assayed by a procedure previously described.[2] Samples were incubated in 25-ml Erlenmyer flasks at 37° and were removed at 30, 45, 60 and 75 min. Activity was recorded as change in absorbancy per min per mg protein over the linear portion of the curve. Zero time tissue blanks and serotonin control samples were also run.

The oxidation of dopamine and norepinephrine by tyrosinase was assayed spectrophotometrically by following the increase of absorption at 310 mμ according to a previously described procedure.[5] Incubation of serotonin with tyrosinase produced no absorption at this wavelength.

The autoxidation of dopamine and norepinephrine was again followed spectrophotometrically by an increase of absorption at 310 mμ. The rate of oxidation was accelerated by the addition of 0·01 N NaOH. All solutions were made with distilled water for the autoxidation experiments.

Physical demonstration of dopamine–serotonin complex

Changes in serotonin solubility. A saturated solution of serotonin was prepared in distilled water so there would be an appreciable amount of serotonin remaining undissolved. The concentration of serotonin which was found to meet these requirements was 5×10^{-2} M. To this solution was added enough dopamine powder to give an equimolar concentration. The effect of the addition of dopamine on the undissolved serotonin was recorded after visual observation of the precipitate.

Reaction with diazotized o-toluidine. Solutions (10^{-2} M) of serotonin and dopamine were prepared in 0·1 M $NaPO_4$ buffer (pH 6·8) and in pure distilled water. Solutions of equimolar amounts of both dopamine and serotonin were also prepared to allow the complex to form. Several drops (10^{-7} moles) of the dopamine and serotonin control solutions as well as the mixed solution were spotted on filter paper and sprayed with diazotized *o*-toluidine. After spotting, the papers were viewed under u.v. light of long wavelength in the model C Chromato-vue manufactured by Ultra-violet Products, Inc. Diazotized *o*-toluidine was prepared fresh each day as follows: 0·1 ml of *o*-toluidine was dissolved in 1 ml of concentrated HCl and 49 ml of distilled water. An equal volume of freshly prepared 0·2% sodium nitrite was slowly added to the above solution. Immediately before use the diazotized *o*-toluidine was mixed with an equal volume of 10% sodium carbonate.

Melting point determinations with the complex. Supersaturated solutions of seroto-

nin, dopamine and the serotonin–dopamine mixture (0·2 M) were allowed to crystallize. The crystals were collected, observed under the microscope and examined for melting points.

Inhibition of catecholamine autoxidation. Dopamine (10^{-3} M), norepinephrine (10^{-3} M) and epinephrine (10^{-4} M) autoxidations were initiated by the addition of 0·01 N NaOH and the reaction followed spectrophotometrically at 310mμ using a Beckman DK-2 recording spectrophotometer. The compounds tested for inhibition were added to give a final concentration of 10^{-3} M.

RESULTS

Enzyme-catalyzed reactions

Figure 1 shows the inhibitory effect of 5×10^{-3} M serotonin on pigment formation from dopamine (10^{-2} M) by the rat brain preparation. This concentration of serotonin caused a 53 per cent inhibition. Since the serotonin used in these experiments was the creatinine sulfate salt, it seemed essential to resolve that the creatinine sulfate itself had no effect on pigment formation. As seen in Fig. 1, the addition of 5×10^{-3} M

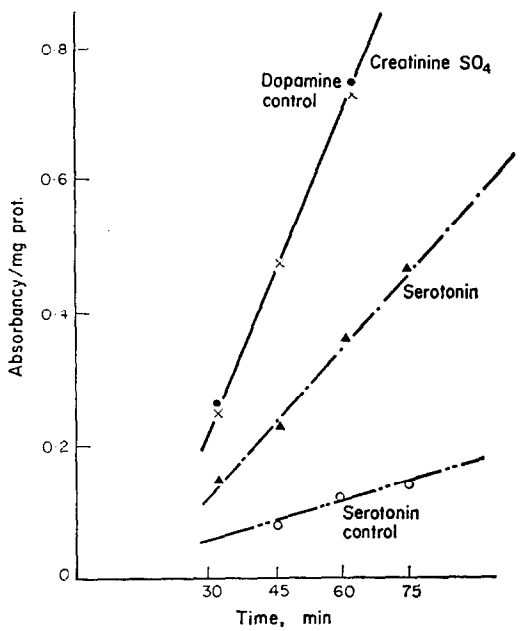


FIG. 1. Effect of serotonin on pigment formation from dopamine in brain preparations. The incubation medium contained 10^{-2} M dopamine, 5×10^{-3} M serotonin and 0·8 ml brain preparation (0·8 to 1·0 mg protein) in a total volume of 2·0 ml of 0·1 M $NaPO_4$ buffer, pH 6·8. The data represent the averages of three experiments.

creatinine sulfate had no effect on the enzyme activity. The contribution of serotonin to pigment formation was negligible compared to that formed from dopamine. Figure 2 is an inhibitor curve showing the variation of fractional inhibition (i) with the concentration of serotonin (pI) for the brain preparation. This curve exhibits the characteristics seen when an inhibitor reacts with the substrate; namely, the inflection point of the curve falls in the region where the inhibitor concentration is comparable

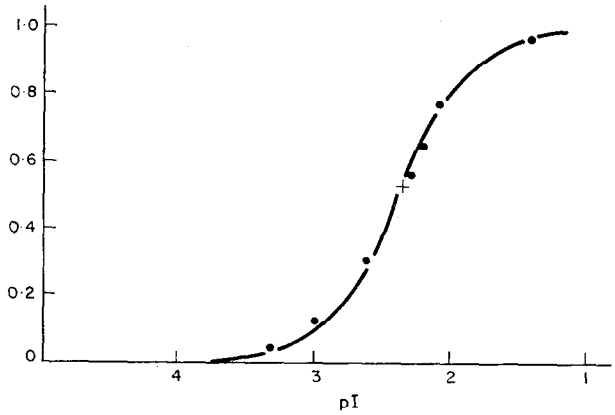


FIG. 2. Variation of fractional inhibition (i) with serotonin concentration (pI) in brain preparations The dopamine concentration was 10^{-2} M and the enzyme concentration 0·8 to 1·0 mg protein as described in Fig. 1. Serotonin concentration was varied.

to substrate concentration. Also, it was found that the ratio $(I_t)90/(I_t)_{10}$ equalled 30 and is very close to that expected for complex formation with substrate.[6] In this ratio $(I_t)90$ represents the concentration of serotonin producing 90 per cent inhibition while the $(I_t)_{10}$ is the concentration producing 10 per cent inhibition.

Figures 3 and 4 show the variation of fractional inhibition with serotonin concentration for the tyrosinase reaction. In this reaction, the $(I_t)90/(I_t)_{10}$ was 20 for dopamine, which would be expected for an inhibitor–substrate complex. With norepinephrine, the ratio was approximately 10, again consistent with this mechanism. The inflection point of the dopamine curve is removed from the point where the inhibitor concentration is equivalent to the substrate concentration as anticipated for

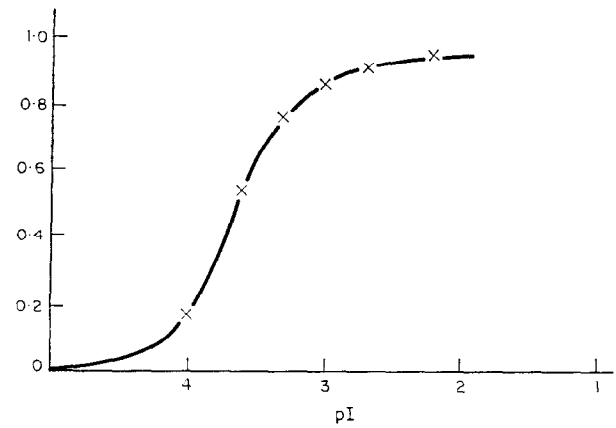


FIG. 3. Variation of fractionational inhibition of tyrosinase oxidation of dopamine with serotonin concentration. The reaction was run at room temperature in a 3·0-ml cuvette with the wavelength set at 310 mμ. The reaction mixture contained 10^{-3} M dopamine, 2 μg of tyrosinase, and varying concentrations of serotonin. The total volume was 3·0 ml in 0·1 M $NaPO_4$ buffer, pH 6·8.

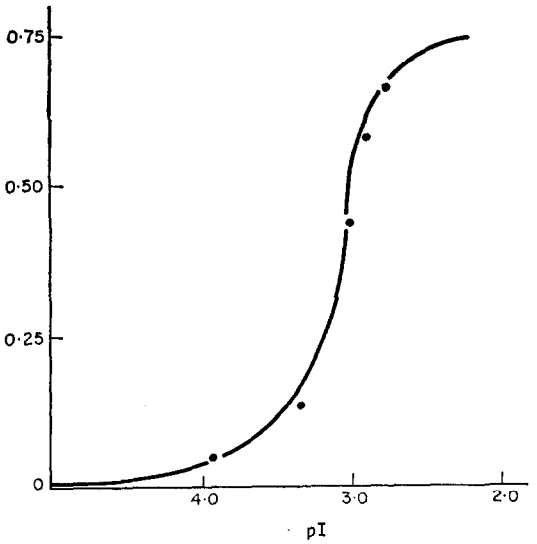


FIG. 4. Variation of fractional inhibition of tyrosinase oxidation of norepinephrine with serotonin concentration. Norepinephrine concentration was 10^{-3} M and the serotonin concentration was varied. The assay procedure was the same as in Fig. 3.

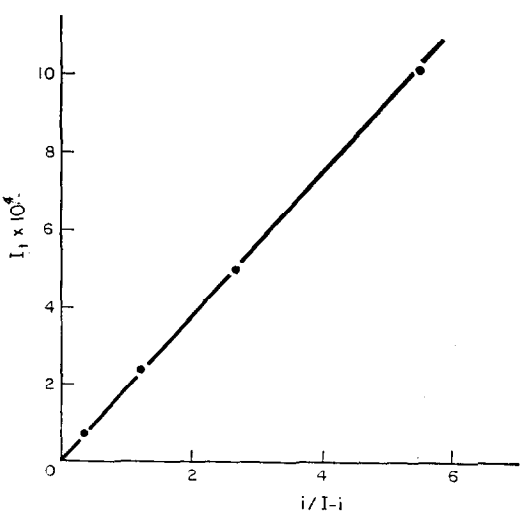


FIG. 5. Inhibitor curve for serotonin effects on tyrosinase-catalyzed oxidation of dopamine.

this mechanism. The tyrosinase reaction appeared to be more sensitive to inhibition than the brain preparation. The explanation for this would appear to be in Fig. 5 where I_t (the amount of inhibitor added) is plotted against $i/_{I-i}$. According to Reiner,[7] in this type of plot, a straight line passing through the origin almost surely represents inhibition of the enzyme by the inhibitor–substrate complex.

Figure 6 shows the corresponding curve with norepinephrine. Although this curve

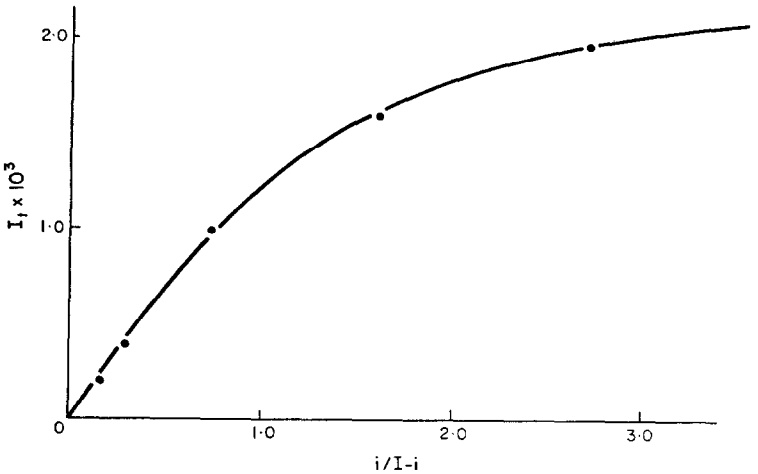


FIG. 6. Inhibitor curve for serotonin effects on tyrosinase-catalyzed oxidation of norepinephrine.

indicates inhibitor–substrate complex formation as a mechanism, it differs from the dopamine curve in that the complex itself does not inhibit the enzyme.

Autoxidation

Figures 7 and 8 show the effect of serotonin on the autoxidation of dopamine and norepinephrine. In both cases, the inhibition of oxidation was a logarithmic function of serotonin concentration. The use of NaOH to accelerate the autoxidation was sufficient to prevent any significant change in the pH of the reaction mixture upon addition of serotonin.

Physical demonstration of serotonin–dopamine complex

Serotonin solubility. Serotonin exhibited greater solubility in equimolar solutions of

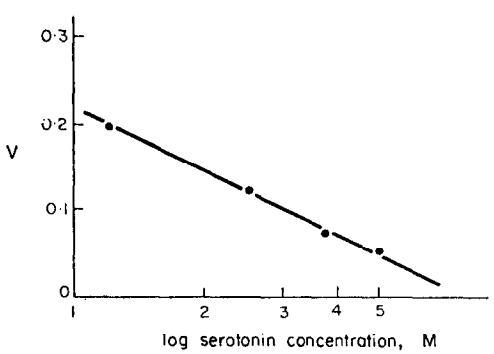


FIG. 7. Inhibition by serotonin of dopamine autoxidation. The reaction was run at room temperaturs in a 3·0 ml-cuvette. Dopamine concentration was 10^{-3} M and the serotonin concentration was varied. The reaction was accelerated by the addition of 0·1 ml of 0·01 N NaOH. The total volume was 3·0 ml in distilled water. V represents the change in absorbancy per minute.

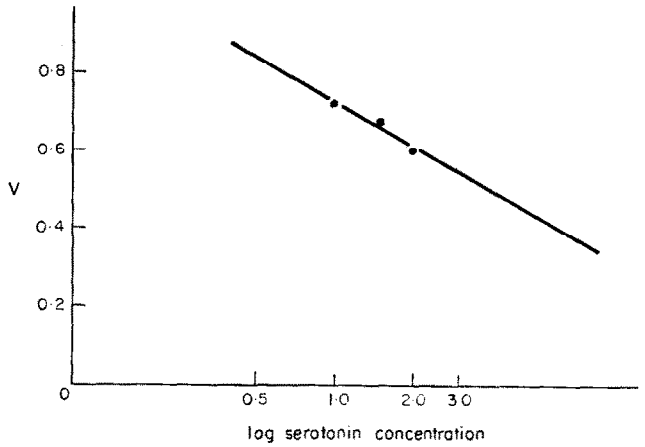


FIG. 8. Inhibition by serotonin of norepinephrine autoxidation. Norepinephrine concentration was $0{\cdot}5 \times 10^{-3}$ M and the serotonin concentration was varied. The assay was the same as in Fig. 7. V represents the change in absorbancy per minute.

dopamine as a solvent than it did in either 0·1 M $NaPO_4$ buffer, pH 6·8, or in pure distilled water which contained no dopamine. This was evidenced by the complete disappearance of the undissolved serotonin when dopamine was added. There was no significant change in the pH of the system after dopamine was added, indicating that the increased serotonin solubility was not pH dependent.

Reaction with diazotized o-toluidine. In the reaction with diazotized *o*-toluidine, the serotonin–dopamine mixture contained an intense pale-yellow fluorescing compound when viewed under long u.v. light. The fluorescence developed within 1 to 2 min and was seen only in the dopamine-serotonin mixture. Neither dopamine nor serotonin alone was found to fluoresce under these conditions. Mixtures of dopamine and creatinine sulfate did not fluoresce. Figure 9 shows the fluorescing band.

Melting point determinations. Table 1 shows the melting points of dopamine, serotonin, the dopamine–serotonin complex and a mixture of serotonin and dopamine crystals ground together. The last sample served as a control to determine what effect

TABLE 1. MELTING POINTS OF CRYSTALS FORMED FROM DOPAMINE, SEROTONIN AND THE DOPAMINE–SEROTONIN MIXTURE*

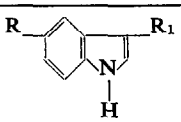
Crystal	Melting point (°)	Comments
Serotonin	220–223	Sharp melting point.
Dopamine	240–248	Samples started to char at 190°. Melting point was not sharp.
Serotonin–dopamine (physically mixed)	215–227	Signs of dopamine charring were observed. Melting point was not sharp.
Serotonin–dopamine (complex)	231–233	Sharp melting point. No signs of charring.

* See Methods section for details.

a simple physical mixture (co precipitation) of serotonin and dopamine crystals would have on the melting points. Serotonin exhibited a melting point of 220–223°. Samples of dopamine alone started to char at approximately 190° and melted at 240–248°. When the two crystals were physically mixed together, the melting point was 215–227°. Signs of dopamine charring were evident in these samples. In contrast to the above samples, the crystals formed from the dopamine–serotonin mixture exhibited a comparatively sharp melting point of 231–233°, with no signs of the charring seen with dopamine alone or in the ground physical mixture of the two compounds.

Interaction of other indoles with dopamine. Since complex formation between serotonin and dopamine led to the appearance of a fluorescing compound when reacted with diazotized *o*-toluidine, this procedure was used as a screening method to detect possible interaction of other indole derivatives with dopamine. Table 2 shows

TABLE 2. STRUCTURE–ACTIVITY STUDIES WITH INDOLE DERIVATIVES*



R	R_1	Fluorescence	Effect on autoxidation of DA
—OH	$-CH_2-CH_2-NH_2$	+	I
—OH	$-(CH_2)_2-N-(CH_3)_2$	+	I
—OH	$-CH_2-COOH$	+	I
$-OCH_3$	$-CH_2-CH_2-NH_2$	—	
$-OCH_3$	$-(CH_2)_2-NH-\overset{\overset{O}{\|}}{C}-CH_3$	—	
None	$-CH_2-CH_2-NH_2$	—	
None	$-CH_2-COOH$	—	
None	None	—	

* Mixtures of the different derivatives with dopamine were made, spotted on filter paper, sprayed with diazotized *o*-toluidine, and observed under long u.v. light for fluorescence. The occurrence of a fluorescent band was graded as positive. The equivalent of 10^{-7} moles was spotted on the paper.

the results of these studies. Serotonin was used as a control in each experiment to confirm the development of fluorescence. Of the indoles tested, only bufotenine and 5-OH indoleacetic acid gave rise to fluorescence. Indole, indole-3-acetic acid, tryptamine, melatonin and 5-methoxytryptamine did not. To confirm that the fluorescence was representative of probable complex formation, these results were correlated with the ability of these compounds to inhibit dopamine oxidation. These results are also seen in Table 2. Only those compounds which produced fluorescence inhibited dopamine oxidation. From these results it appears that the only structural requirement for interaction of the indole derivatives with dopamine is the hydroxyl group on the ring. Alteration of the side-chain did not influence complex formation as long as the 5-hydroxyl group was intact. This can best be seen by the comparison of 5-OH indole acetic acid with indole acetic acid, and serotonin with 5-methoxytryptamine. In both situations the only difference between the compounds is the 5-hydroxyl group.

Structural requirements for the catecholamines. In similar studies, the requirements for the catecholamines were determined. Table 3 records the results. Both epinephrine and norepinephrine gave rise to fluorescence which was consistent with the finding

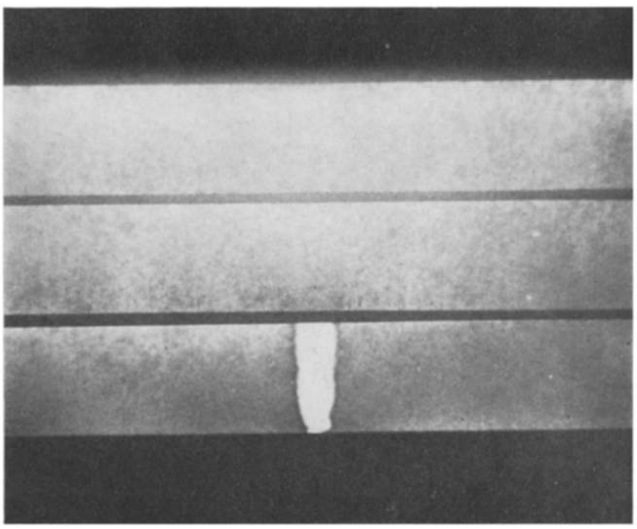
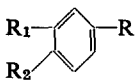


FIG. 9. Fluorescing complex of dopamine and serotonin. See Methods section for details. Upper strip, serotonin control; middle strip, dopamine control; lower strip, dopamine–serotonin complex.

TABLE 3. STRUCTURAL REQUIREMENTS FOR CATECHOLAMINES*



R	R_1	R_2	Fluorescence	Effect of 5-HT on autoxidation
—CH_2—CH_2—NH_2	—OH	—OH	+	I
—CHOH—CH_2—NH_2	—OH	—OH	+	I
—CHOH—CH_2—NH—CH_3	—OH	—OH	+	A
—CH_2—COOH	—OH	—OH	+	
None	—OH	—OH	+	
—CH_2—CH_2—NH_2	None	—OH	−	
—CH_2—$CHNH_2$—COOH	None	—OH	−	
—CH_2—CH_2—NH_2	—OCH_3	—OH	−	
—CH_2—CH_2—NH_2	—OH	—OCH_3	−	
—CH_2—CH_2—NH_2	—OCH_3	—OCH_3	−	
—CH_2—CH_2—NH_2	None	None	−	

* Mixtures of the different compounds with serotonin were made, spotted on filter paper, sprayed with diazotized *o*-toluidine and observed under long u.v. light for fluorescence. The appearance of a fluorescent band was graded as positive. The equivalent of 10^{-7} moles was spotted on the paper.

that serotonin can inhibit the oxidation of these substances. Both 3,4-dihydroxyphenylacetic acid and catechol itself gave rise to a fluorescing band, while the methoxy dopamines did not. This indicated that both of the ring hydroxyl groups were required for interaction with serotonin and that the side-chain had little or no influence. The possibility that the presence of one methoxy group could interfere with the interaction on the other hydroxyl group was considered, but no fluorescence could be detected with either tyramine or tyrosine which contain only one hydroxyl group on the ring. Phenylethylamine, which contains the same side chain as dopamine but lacks the ring hydroxyls, did not give rise to fluorescence.

The requirement for the hydroxyl groups in both series of compounds led us to believe that the complex is formed through hydrogen bonding between these groups. To test this hypothesis, an attempt to interfere with complex formation using urea was made. Urea (8 M) was added to the mixture of dopamine and serotonin, but there was no marked interference with the development of the fluorescing band.

In spite of the fact that there was no clear indication that a π complex was forming, this possibility was again considered. Silver nitrate (up to 2×10^{-2} M) was added to the mixture. Since silver ions form very strong π complexes, it was hoped that the silver would form π complexes preferentially with the aromatic rings and would, therefore, interfere with the formation of the fluorescing band. This did not prove to be the case. If, however, silver ions were added in combination with urea, interference with the fluorescence resulted. The possibility that both hydrogen bonding and π complexing could be involved in the indole amine–catecholamine interaction is being considered. This interpretation is at the present, however, tentative. Further studies are in progress to resolve the actual nature of the complex.

DISCUSSION

Although serotonin proved to be an effective inhibitor of pigment formation from dopamine and norepinephrine by enzyme preparations, the inhibition of autoxidation suggested that interference with these reactions could result from complex formation

with the catecholamines. This led to the kinetic evalation of the enzyme reactions as a means of demonstrating complex formation. With both the brain preparation and tyrosinase, the kinetic data indicated that serotonin was complexing the substrates. In the tyrosinase reaction it appeared that the serotonin–dopamine complex was also inhibiting the enzyme. This was not the case for norepinephrine. This observation proves to be rather interesting in that the interaction of serotonin with the different catecholamines can lead to varying effects. This difference became extremely pronounced with epinephrine where serotonin had a biphasic effect. At low concentrations it accelerated adrenochrome formation whereas at higher concentrations it was inhibitory.[4]

The present studies provide a basis for suggesting a role for serotonin which does not involve neurotransmission. Serotonin could act to modulate the activities of the catecholamines on a purely molecular level through complex formation. Such a relationship between serotonin and dopamine might be suggested by the distribution of these compounds in certain brain nuclei. The substantia nigra and caudate nucleus contain relatively high concentrations of dopamine and serotonin. Laverty *et al.*[8] examined the subcellular distribution of dopamine relative to other amines in the caudate and found that most of the dopamine was in the soluble fraction, whereas serotonin was bound. In view of the suspected transmitter role of dopamine in this nucleus, this is a convenient arrangement in that bound serotonin should have little influence on dopamine, whereas upon its release, complex formation would result in the modulation of dopamine's action. This type of relationship between serotonin and dopamine in the substantia nigra would also have interesting possibilities. That dopamine may serve as the precursor of neuromelanin found in this area of the brain has recently been discussed.[2, 9] An excessive release of, or a failure to bind, serotonin could then lead to a reduction of melanin. Indeed, a depletion of melanin in the substantia nigra occurs in human Parkinsonism,[9] a disease in which there are alterations in both dopamine and serotonin levels.

Acknowledgements—Preliminary reports on parts of this work were presented at the Spring, 1967 meeting of the Federation of American Societies for Experimental Biology, and at the Fall meetings of the American Society for Pharmacology and Experimental Therapeutics. Parts of this work were submitted by J. C. J. to the Department of Biochemical Pharmacology of the College of Pharmacy Rutgers, The State University, in partial fulfillment of the requirements for the Master of Science Degree.

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