SERUM VASOCONSTRICTOR (SEROTONIN)

V. THE PRESENCE OF CREATININE IN THE COMPLEX. A PROPOSED STRUCTURE OF THE VASOCONSTRICTOR PRINCIPLE

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In the fourth paper in this series (1) the isolation of the substance believed to be responsible for the marked vasoconstrictor activity of serum was reported. This substance was characterized as an indole derivative from its ultraviolet absorption spectrum and its behavior in various color and other chemical reactions. The analytical data agreed well with the empirical formula \( C_{14}H_{21}O_3N_6 \cdot H_2SO_4 \). The titration curve in aqueous solution revealed two titratable groups with \( pK' \_1 \) of 4.9 and \( pK' \_2 \) of 9.8. Both of these groups were believed to be basic in nature, and the \( pK' \) value of the weaker suggested an acylated guanidine structure. Indeed, the value was very close to that reported in the literature for creatinine, while the formation of ammonia on treatment with alkali and the presence of one \( N \)-methyl grouping suggested that a creatinine residue might well be present in the molecule.

The fact that only exceedingly small quantities of pure substance were available led to an investigation of this possibility by colorimetric means, and it was quickly determined that serotonin sulfate responded positively to both the Jaffe alkaline picrate reagent and the Benedict alkaline 3,5-dinitrobenzoic acid reagent, with a color intensity equal (within an experimental error of about 10 per cent) to that of creatinine on a molar basis. Benedict's study (2) of the specificity of the alkaline 3,5-dinitrobenzoic acid reagent with respect to creatinine showed that derivatives and closely related compounds such as glycocyamidine could be readily distinguished by measuring both the intensity of color and the rate of color development. Comparison of serotonin sulfate with creatinine in this fashion again revealed almost identical behavior.

It then became necessary to investigate the question of whether a creatinine-like residue was actually part of the molecular structure of serotonin, or whether, as now seemed more likely, the entity isolated and designated as serotonin sulfate was not in reality a complex containing an indole base, creatinine, and sulfuric acid.

The behavior of serotonin on a paper chromatogram developed with butanol showed that the latter was indeed the case, for the creatinine chromogen was completely separable from the indole chromogen.
The presence of creatinine was then directly confirmed by isolation of its picrate. In addition, the crystalline picrate of the indole base was obtained for the first time. Elementary analysis of the latter confirmed the empirical formula \( \text{C}_{10}\text{H}_{14}\text{O}_2\text{N}_2 \) obtained by deducting that of creatinine \( \text{C}_{14}\text{H}_{10}\text{N}_3\) from that of the complex \( \text{C}_{14}\text{H}_{21}\text{O}_3\text{N}_5\).

**EXPERIMENTAL**

Melting points were determined on the micro hot stage and are uncorrected.

*Color Reactions*—The serotonin sulfate complex was compared with creatinine for intensity of color per mole in both the alkaline dinitrobenzoic acid reaction of Benedict and the alkaline picric acid reaction of Jaffe.

The method used for the dinitrobenzoic acid reaction was that described by Benedict and Behre (2). To 5.0 ml. of unknown in aqueous solution were added 1.0 ml. of 5 per cent ethanolic 3,5-dinitrobenzoic acid and then 1.0 ml. of 5 per cent aqueous sodium hydroxide. After standing 15 minutes at room temperature, the optical density was determined at \( \lambda = 5400 \text{ A} \) with a Coleman model 11 universal spectrophotometer and No. 6-304B cuvettes.

The picric acid reaction was carried out as follows. To 5.0 ml. of unknown solution were added 2.5 ml. of an alkaline picric acid solution made by adding 1.0 ml. of 10 per cent aqueous sodium hydroxide to 5.0 ml. of saturated aqueous picric acid. After standing at room temperature for 20 minutes, the color was determined with the Coleman instrument at \( \lambda = 5200 \text{ A} \).

The results (Table I) show that serotonin complex gives a molar color development almost equal to that of creatinine by both methods.

The rates of color formation with Benedict’s reagent of serotonin sulfate complex and creatinine (Table II) are almost identical. In this experiment the ratio of color per mole of serotonin complex to color per mole of creatinine is 0.94.

*Paper Chromatography*—40 \( \gamma \) of serotonin sulfate complex in 0.01 ml. of water and 10 \( \gamma \) of creatinine in 0.01 ml. of water were placed on Whatman No. 1 filter paper and developed with butanol saturated with water by downward irrigation for 17 hours at room temperature. Two strips of the serotonin and one of the creatinine were run simultaneously. After drying in air, one of the serotonin strips (C) was cut off and sprayed with Ehrlich’s reagent.\(^1\) The remaining two strips (A and B) were

\(^1\) The spraying reagent was prepared by adding 6 ml. of butanol to 1 ml. of a solution containing 1 gm. of \( p \)-dimethylaminobenzaldehyde in a mixture of 30 ml. of 95 per cent ethanol and 30 ml. of concentrated hydrochloric acid (5).
sprayed with Benedict's reagent.² The results (Fig. 1) show that the indole portion of the complex (C) was completely separated from the creatinine portion (B) and that the latter migrates with almost the same velocity \(R_F = 0.15\) as creatinine \(R_F = 0.16\). The colors obtained

### Table I

**Comparison of Intensity of Color Produced by Serotonin Sulfate Complex and Creatinine in Benedict and Jaffe Reactions**

<table>
<thead>
<tr>
<th></th>
<th>Benedict alkaline</th>
<th>Jaffe alkaline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serotonin sulfate complex, 33 γ</td>
<td>Creatinine, 10 γ</td>
</tr>
<tr>
<td>Color density</td>
<td>0.033</td>
<td>0.039</td>
</tr>
<tr>
<td>Color per mole serotonin sulfate complex</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>Color per mole creatinine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The quantity of serotonin sulfate complex was determined from the ultraviolet absorption at 2750 A in the Beckman spectrophotometer, with 10 mm. cells. The calculation was made from the formula, \(\gamma\) per ml. = \((\text{density} \times 100)/1.43\), for densities between 0.2 and 0.6.

### Table II

**Rate of Color Development with Benedict’s Reagent of Serotonin Sulfate Complex and Creatinine**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Serotonin sulfate complex, 205 γ</th>
<th>Creatinine, 50 γ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Color density</td>
<td>Per cent maximum color</td>
</tr>
<tr>
<td>1</td>
<td>0.084</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>0.148</td>
<td>64</td>
</tr>
<tr>
<td>5</td>
<td>0.182</td>
<td>78</td>
</tr>
<tr>
<td>10</td>
<td>0.218</td>
<td>94</td>
</tr>
<tr>
<td>15</td>
<td>0.231</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>0.232</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>0.220</td>
<td>95</td>
</tr>
</tbody>
</table>

*See foot-note to Table I.

with both Ehrlich’s and Benedict’s reagents are initially violet. The color developed with Benedict’s reagent fades to pink and then disap-

² The spraying reagent was prepared by adding 3 ml. of butanol to a solution of 2 ml. of 5 per cent ethanolic dinitrobenzoic acid plus 1 ml. of 5 per cent aqueous sodium hydroxide.
pears. A pale brown color slowly develops at the position of the indole base (strip B). This reaction is believed to be responsible for the less rapid fading of the color obtained with serotonin sulfate complex as compared with creatinine (Table II).

Isolation of Picrates—9.4 mg. of serotonin sulfate complex were dissolved in 0.5 ml. of water by slight warming and 0.5 ml. of saturated aqueous picric acid was added. A faint reddish coloration and the development of turbidity were observed. The solution was warmed until it became almost clear, and it was then allowed to cool slowly. Beautiful orange-red needles began to separate as crosses which became tufted and then developed through sheaves into perfect rosettes. On standing overnight a second kind of crystal made its appearance, dense yellow rosettes of prisms. The crystals were centrifuged, washed with a minimum quantity of water, and dried by evaporation on the water pump. The four dense yellow clusters (3.5 mg.) were separated mechanically from the fluffy orange-red needles (4.3 mg.).

The yellow clusters were recrystallized from a small quantity of water and separated as individual stout yellow prisms melting with decomposition at 207–210° when heated at a rate of 2° per minute. Creatinine picrate was prepared from an authentic sample of creatinine (Pfanstiehl) and was obtained on recrystallization from water as typical, long, straw-like, yellow needles which melted, under identical conditions, at 208–211° with decomposition. The mixed melting point was 207–210°.
In a qualitative spot test with Benedict's reagent, both samples showed identical behavior, forming an orange-red color at the site of the crystal which developed into a violet-brown color in the solution.

It is believed that the evidence favoring the identity of the two picrates outweighs the observed difference in crystal habit.

The orange-red needles of the indole picrate were recrystallized twice from water. The melting point determination was complicated by several distinct transitions, unaffected by further recrystallization. Fusion began at 105–110°. Some crystals melted completely, but the mass slowly became deep red in color. Resolidification of the melted crystals then began and was essentially complete by 130°. The deep red color paled from 165–175°, and complete fusion with decomposition occurred at 185–188°. The rate of heating during transitions and melting was 2° per minute.

These crystals gave no color with Benedict's reagent, but with Ehrlich's and Folin's reagents qualitative spot tests were positive. The analytical sample was dried at 80° over phosphorus pentoxide in vacuo to constant weight.³

\[
\text{C}_{10}\text{H}_{10}\text{O}_{2}\text{N}_{2} \cdot \text{C}_{6}\text{H}_{5}\text{O}_{2}\text{N}_{2}. \text{ Calculated. C 45.39, H 4.05, N 16.55} \\
\text{423.3} \text{ Found. " 45.65, " 4.23, " 16.95}
\]

³ Analyses by Dr. A. Elek, Los Angeles.
Ultraviolet Absorption Spectrum—The ultraviolet absorption spectrum of serotonin sulfate complex was reported (1) to be essentially the same with regard to maxima and general contour in aqueous solution at pH 3.5 and 10.3. It has now been found that at pH 11.6 (Beckman pH meter) the absorption spectrum shows a remarkable shift of the second maximum (Fig. 2). The significance of this change will be discussed below. Absorption measurements were made with a Beckman model DU quartz spectrophotometer.

DISCUSSION

The identification of creatinine in the serotonin sulfate complex confirms the molecular weight of 405 for this complex, since the dissociation constant characteristic of this substance is in accord with that obtained from the theoretical dissociation curve calculated on the assumption of this molecular weight (1). Additional evidence favoring this value is obtained from the molecular extinction at 2750 A which agrees well with that expected for absorption of indoles. There is, then, little doubt that the empirical formula of the indolic base portion of the complex (which is also, presumably, responsible for the pharmacological activity) is C_10H_14O_2N_2. The molecular complexity of the vasoconstrictor principle is thus not much greater than that of epinephrine.

If we subtract the formula for the indolyl group (C_8H_6N), the number of atoms which must still be put into place is very few, C_2H_8O_2N. The number of suitable structures which can be written is therefore quite limited, and before discussing the possibilities, it might be well to recapitulate those properties which an acceptable structure must satisfactorily explain.

The most important of these is the ultraviolet absorption spectrum which deviates in contour quite markedly from that found for such other widely occurring indoles as tryptophan, tryptamine, and indican. The shift in the second maximum from 2930 A to 3230 A which occurs on raising the pH to 11.6 (Fig. 2) and which is not observed at pH 10.3 (1) suggests the presence of a weakly acidic function. The position of the maximum at 2750 A, which remains unchanged during these shifts in pH, is consistent with indolic absorption. Clearly then, the maximum at 2930 A is attributable to a second absorbing group. It would be difficult to locate such a group in a side chain which maximally contains C_2H_8O_2N, especially since this empirical formula is already above the theoretical degree of saturation. The assumption of a second nuclear substitution of the indole must therefore be made, and a phenolic grouping would most easily account for the observed facts. This conclusion gains further support from the color reaction with the Folin-Ciocalteau reagent,
which indicates the presence of a second reducing group besides the indole (1). It might be remarked that several attempts to detect the odor of indole on micro zinc dust distillation of serotonin sulfate complex met with no success, although positive results were easily obtainable with compounds containing a simple indole nucleus.

With regard to the location of this phenolic group, the intensity and rate of color development in the quantitative Hopkins-Cole reaction (1) indicate that positions 1 and 2 of the indole nucleus are unsubstituted. Comparison of both the Hopkins-Cole reaction and the ultraviolet absorption with that of indican\(^4\) rules out position 3. Of the remaining four positions in the benzene ring, biological analogy to such naturally occurring compounds as physostigmine and, more importantly, bufotenine (I), strongly favors position 5. As has been pointed out by Wieland et al. (3), no simple indole substituted in positions 4 or 7 has ever been found in nature. Although these monohydroxyindoles have never been synthesized,\(^5\) the methoxy compounds are known. Pruckner and Witkop (4) have published the ultraviolet absorption spectra of 5-methoxy- and 6-methoxyindole dissolved in alcohol, in connection with their studies of yohimbine. The curves are quite different from each other, and that of the 5-methoxy compound shows the same contour and almost the identical molecular extinctions of the two maxima observed with serotonin. Although the positions of the maxima of 5-methoxyindole are displaced 60 Å toward the shorter wave-lengths, the similarities lend considerable weight to the argument favoring the 5 position for the phenolic substituent.

If the assumptions made are correct, the structure would now stand at II. It can be readily seen that two possibilities can explain such an anomalous side chain. The first would require that the empirical formula be in error by two hydrogen atoms, and the second would postulate the presence of 1 mole of water of crystallization. With regard to the first, the three analyses are consistent in showing hydrogen values which are higher than the theoretical. An excess of two hydrogen atoms in the empirical formula would therefore mean that the analytical results were

\[\text{(I) } R = \text{CH}_2\text{CH}_2\text{N(CH}_3\text{)}_2\]
\[\text{(II) } R = \text{C}_2\text{H}_5\text{ON}\]
\[\text{(III) } R = \text{CH}_2\text{N(CH}_3\text{)}_2\]
\[\text{(IV) } R = \text{CH}_2\text{CH}_2\text{NH}_2\]

\(^4\) Unpublished experiments.

\(^5\) The synthesis of the monohydroxyindoles has been described in a recent paper (Beer, R. J. S., Clarke, K., Khorana, H. G., and Robertson, A., J. Chem. Soc., 1605 (1948)).
in error for hydrogen by 12 to 18 per cent, a possibility which seems unlikely.

The second alternative, namely, the presence of water of crystallization, is believed to be more consistent with the facts. Although such hydrate water was not detectable in the complex despite prolonged drying at $110^\circ$, hydrates which do not lose their water below the decomposition point are known to occur. In fact, with the compound most closely related to that under consideration, namely, bufotenine (I), this phenomenon led to an incorrect empirical formulation on the basis of analyses of the oxalate salt (3). Inasmuch as both serotonin derivatives which were analyzed were crystallized from water, the probability of the presence of hydrate water is high. It might be noted further that the sulfate complex effervesces slightly on decomposing (1), while the picrate shows a decided transition at 105–110°, both observations being explainable by the presence of hydrate water.

If this assumption is made, the side chain would be $C_2H_5N$. With the exception of the presence of a basic group dissociating with a $pK'$ of 9.8 (1), there is no evidence pertaining to this grouping. However, analogy to those compounds known to exert such powerful pharmacological effects presents an exceptionally strong argument in favor of a simple ethylamine side chain. Tentatively, then, the constitutional formula of 5-hydroxytryptamine (IV) may be assigned to this vasoconstrictor principle isolated from beef serum.

It should be mentioned that, besides the necessity for assuming the presence of water of crystallization, one other property of the substance is not in accord with the proposed structure, and that is the failure to obtain a red or violet color with ninhydrin-sodium acetate. This reaction is positive with tryptamine and most other primary amines. However, the evidence against a secondary amine formed from the available atoms is much stronger, namely, the presence of only 1 $N$-methyl group in the complex (1), which must be attributed to the creatinine, and the rate and intensity of color formation in the Hopkins-Cole reaction. The color development is exceptionally slow and the intensity very weak with gramine (III), but they are comparable to that of tryptophan with serotonin.

Since a chemical method for the specific determination of serotonin is, at present, one of the most pressing problems connected with its further study, attention may be called to a property characteristic of this substance which may well serve as the basis for such a method. As can be seen from Fig. 2, the absorption of light at 3230 A increases sharply on raising the pH to 11.6. The location of this maximal increase, displaced as it is from the position of protein and other commonly encountered absorptions, as well as its dependence on pH should make it possible to determine serotonin specifically in amounts greater than 10 $\gamma$. 
In the interest of simplicity, a further revision in nomenclature is proposed. The trivial name serotonin should be reserved for the pharmacologically active indole base. The complex would then be serotonin creatinine sulfate, and the second derivative, reported in this paper, serotonin picrate.

The serotonin creatinine sulfate used in this study was isolated in the laboratories of the Cleveland Clinic Foundation. The author wishes to express appreciation to Dr. Irvine H. Page for permission to retain this material. Grateful acknowledgment is also made to Dr. Hans T. Clarke for helpful discussions and assistance with the preparation of this manuscript.

**SUMMARY**

The crystalline vasoconstrictor substance isolated from beef serum has been shown to be a complex composed of equimolar parts of creatinine, sulfuric acid, and an indole derivative. The latter was converted into its picrate, analysis of which confirmed the empirical formula $\text{C}_{10}\text{H}_{14}\text{O}_{2}\text{N}_{2}$ obtained by deducting creatinine ($\text{C}_{4}\text{H}_{10}\text{Na}$) from the formula of the complex ($\text{C}_{14}\text{H}_{21}\text{O}_{5}\text{N}_{5}$). Evidence based on color reactions and ultraviolet absorption spectra indicates the presence of a 5-hydroxyindole nucleus in the structure. The constitutional formula of 5-hydroxytryptamine ($\text{C}_{10}\text{H}_{12}\text{O}_{2}\text{N}_{2}$) has been tentatively proposed for this active principle.

A recommendation has been made to reserve the name serotonin for this indole amine rather than the previously isolated complex.

The basis for a simple specific method to determine serotonin by ultraviolet absorption spectrophotometry has been suggested.

**BIBLIOGRAPHY**