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THE BINDING OF SEROTONIN IN BRAIN: A STUDY *IN VITRO* OF THE INFLUENCE OF PHYSICOCHEMICAL FACTORS AND DRUGS

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Abstract—The binding of exogenous serotonin-^{14}C to a subcellular fraction (P_2) rich in nerve-endings from guinea pig brain was dependent upon the composition of the incubation medium, pH, temperature, concentration, and time. Further fractionation of the P_2 fraction by sucrose density methods showed that most of the binding was associated with the nerve-ending subfraction. Little or no binding occurred in the microsomal and mitochondrial fractions.

The ruptured nerve-ending, synaptic vesicle, and supernatant fractions were prepared from the P_2 fraction by water treatment. At 37°, only 30 per cent of the original binding activity in the intact P_2 fraction was recovered in these three fractions, whereas at 2°, 80 per cent was found.

The system *in vitro* was relatively specific for the binding of serotonin, since considerably less labeled histamine, tryptophan or norepinephrine was bound by the nerve-ending subfraction.

The antidepressants, imipramine and iprindole (Wy-3263), reduced the binding of serotonin-^{14}C by more than 50 per cent in the nerve-ending subfraction. D-lysergic acid diethylamide (LSD) produced a 21 per cent reduction. Reserpine and iproniazid did not affect the binding.

IT HAS BEEN postulated[1] that serotonin (5-hydroxytryptamine,5-HT) is a chemical mediator in the central nervous system. If this is so, then one would expect that its distribution and storage in brain should be regulated by relatively specific mechanisms. The active transport of this substance has been demonstrated in some peripheral tissues[2, 3] but not in the brain.[4, 5] In addition, neither the factors influencing the binding of serotonin nor the precise sites of binding have been satisfactorily clarified.

The sites of binding of endogenous serotonin have been investigated by Michaelson and Whittaker[6] and by Zieher and De Robertis,[7] and it now seems well established that at least half of the endogenous serotonin is bound by the nerve-ending fraction. Zieher and De Robertis[7] also showed that, after rupture of the nerve-endings, serotonin is found mainly in the fraction containing the ruptured nerve-endings and not in the synaptic vesicle fraction. The findings concerning other sites of binding are conflicting. Zieher and De Robertis,[7] Ryall,[8] and Gillis *et al.*[9] reported that a significant portion of endogenous serotonin was bound by the microsomal fraction, whereas Michaelson and Whittaker[6] and Giarman and Schanberg[10] found little serotonin present in this fraction.

Finally, studies[4, 9, 11–16] *in vitro* have shown that exogenous serotonin is bound to isolated fractions of brain containing nerve-endings. However, there are a number

of conflicting findings among these reports as to the factors which influence binding (e.g. time of incubation, pH, temperature) and as to the effect of drugs on binding (see Table 2). On the one hand, some authors[4, 9, 15] have concluded that the binding of exogenous serotonin is nonspecific or based on nonspecific ion-exchange. On the other hand, Marchbanks[12] has suggested that there is at least one type of binding of serotonin which is relatively specific to nervous tissue. Marchbanks took the inhibition of serotonin binding to nerve-endings (produced by pharmacological antagonists such as LSD*) as evidence that this type of binding was connected with the central pharmacological function of serotonin. However, Marchbanks did not evaluate how some of the physicochemical parameters (such as substrate specificity, pH, temperature) affect the binding.

To clarify some of these conflicting findings, we have systematically examined the effect of a number of physicochemical factors on the binding of serotonin. We shall describe an isolated nerve-ending system which, with respect to both the substrate and the physicochemical parameters, possesses a relatively high degree of specificity for the binding of serotonin.

METHODS

Tissue preparation and fractionation. The P_2 fraction, rich in nerve-endings, was prepared from a guinea pig brain homogenate according to the fractionation procedure of Whittaker *et al.*[17] Further subfractionation of the P_2 fraction to obtain nerve-endings was carried out by the methods of Whittaker[18] (with the Spinco 40 rotor in the more routine drug studies), De Robertis *et al.*[19] (for preparation of "cholinergic" and "noncholinergic" nerve-endings), and Michaelson and Whittaker.[20] These procedures also yield mitochondrial and membrane fractions. The microsomal, synaptic vesicle, and ruptured nerve-ending particles were obtained by the methods of Zieher and De Robertis[7] and De Robertis *et al.*[21]

Equilibrium dialysis. The supernatant fraction resulting from the preparation of the ruptured nerve-ending and vesicle fractions was tested for the binding of serotonin by equilibrium dialysis at 2°. Ten ml of the supernatant (derived from treatment of 3·3 g of brain) was added to a dialysis bag, and serotonin-^{14}C (final concentration 1×10^{-7} M) was added to 400 ml of the external buffered medium (described below). The external fluid was stirred slowly with a magnetic bar. After equilibrium was reached (24 hr), the amount of radioactivity was determined in the internal and external fluids (as described below).

Experimental design and incubation procedure. Aliquots of the particulate fraction under investigation were suspended in 3 ml of buffered medium (0·01 M potassium phosphate–0·15 M KCl, pH 8·0). Each aliquot was derived from 0·9 g of brain. Immediately before incubation, 2·1 μg/ml of 5-hydroxy-3-indolyl (ethyl-2-amine-1-^{14}C) creatinine sulphate monohydrate (Nuclear-Chicago) was added. The final concentration of serotonin-^{14}C was 5×10^{-6} M. The sp. act. of the serotonin-^{14}C was 39·6 mc/m-mole and 495 cpm was equivalent to 0·001 μg serotonin. The incubation period was 5 min at 37° unless otherwise noted. Control values were obtained as follows. Aliquots of homogenates were maintained at 2° during the incubation period, after which the isotope was added, and these samples along with the incubated ones

* D-lysergic acid diethylamide.

were immediately centrifuged. The resulting pellets were washed twice (unless otherwise stated) with a 0·2% solution (w/v) of nonradioactive serotonin dissolved in buffer. This procedure was designed to remove extraneous and loosely bound serotonin-^{14}C. The washed pellets were extracted with 0·4 N perchloric acid and filtered. The amount of radioactivity in the clear filtrates was determined by liquid scintillation counting and was corrected by the method of internal standardization. In the pH studies, the pH of the medium was adjusted before the particulate fraction was suspended.

Control values and calculations. Control values are a measure of the binding which occurred after incubation and during the subsequent centrifugation steps while the isotope was still in contact with the particulate fraction (25 min). By subtracting the control values from the incubated values, one obtains a figure representing the amount of binding which occurred during the 5-min, 37° incubation period.

Thin-layer chromatography (TLC). The bound serotonin and its major metabolite, 5-hydroxyindoleacetic acid (5-HIAA), were extracted from the P_2 fraction with 80% methanol–20% water, filtered, and the clear filtrate applied to either of two different thin-layer systems. The first consisted of acid alumina (Woelm) plates and employed *n*-propanol:0·1 N ammonium sulfate:0·1 N sodium phosphate (pH 6·8) (4:120:200) as the solvent. The second consisted of silica gel plates and used cyclohexane:ethanol: ethylacetate:glacial acetic acid (160:160:80:100) as the solvent. The latter plates were prepared by mixing 30 g Fisher silica gel, 10 g Silica TLC-7 (Mallinckrodt), 60 ml of 1·6% (w/v) starch binder, and 20 ml water. After the plates had been developed, the appropriate areas (determined by non-radioactive standards) were scraped into scintillation vials for the determination of serotonin and 5-HIAA. Both systems gave complete separation of the two compounds.

Drug studies. Drugs were added to the buffered medium in a final concentration of 2×10^{-4} M (except reserpine, which was added at 5×10^{-6} M, since it forms a saturated solution in phosphate buffers at this concentration). The pH was checked and if necessary re-adjusted to 8·0. The P_2 fraction was then suspended in the buffered medium containing the drug and serotonin-^{14}C was added. In each experiment, four particulate aliquots were run together, i.e. drug-treated and untreated both at 37° and 2°. After the 5-min incubation and the two washes, the B fraction (nerve-ending) was obtained from the P_2 fraction according to the method of Whittaker,[18] with the Spinco 40 rotor. The amount of radioactivity associated with this fraction was determined as previously described.

Inhibition of monoamine oxidase. The activity of this enzyme was inhibited by s.c administration of iproniazid (100 mg/kg) to the animals 16 hr before the experiment. All experiments, unless otherwise stated, were run with iproniazid-pretreated animals. The endogenous serotonin concentration in the P_2 fraction was determined by the method of Wise.[22]

RESULTS

Fate of the bound isotope and the effect of iproniazid pretreatment. Without iproniazid pretreatment, the amounts of serotonin and 5-HIAA bound to the P_2 fraction (after 5-min incubation) were 41 and 52 per cent respectively. After pretreatment with the monoamine oxidase inhibitor, the relative amounts became 92 and 4 per cent. The percentages reported are based on the total amount of isotope applied at the origin. In

the nerve-ending or B fraction, iproniazid did not affect the amount of bound isotope (see Drug studies in this section and Fig. 8).

The effect of time, temperature, and pH on serotonin binding. The data in Fig. 1 indicate that the binding of serotonin to the P_2 fraction increases linearly for 5 min, then decreases slightly during the next 25 min.

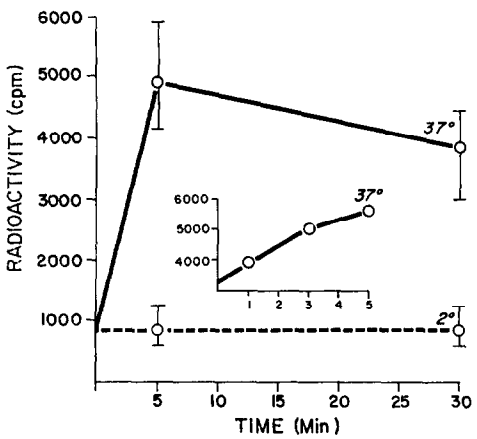

FIG. 1. Rate of binding of serotonin-^{14}C as a function of time by the P_2 nerve-ending fraction of guinea pig brain. Insert shows the rate of binding between 0 and 5 min (in this experiment larger aliquots were used in the radioactive determinations). Solid line, incubations at 37° in phosphate–KCl buffer (pH 8·0); broken line, control levels at 2° (see methods). Vertical bars indicate the range (N)=4.

When a 5-min incubation period was used in order to estimate initial binding rates, the Q_{10} value for the binding process between 22° and 32° was 1·9. No evidence of an inflection point in the temperature curve between 10° and 39° was detected.

The results in Fig. 2 show the effect of pH on serotonin and norepinephrine-^{14}C binding to the P_2 fraction. The P_2 fraction (section A) had a pH optimum at about 9·0 when it had been prepared from animals not pretreated with iproniazid. The control preparations had no pH optimum. When the animals had been pretreated with iproniazid, no decrease in binding between pH 9 and 10 was evident. The binding of norepinephrine-^{14}C was not greatly affected by pH (section B). The two experiments represented in C and D were designed for another purpose and are incomplete, but do indicate the effect of media other than phosphate buffer on serotonin binding. In phosphate buffer, the control values were relatively low and the binding at 37° was several times greater than at 2°, which was not the case in the other two systems.

The ionic form of serotonin was investigated by electrophoresis (cellulose acetate strips, 0·05 M phosphate buffer) at pH 8, 9, and 10. Although serotonin was in the cationic form between pH 8 and 10, a significant amount of other forms occurred at pH 10, as indicated by a decrease in migration (50 per cent of that at pH 9). At pH 9 and 10, trace amounts of material also remained at the origin, presumably due to degradation.

Serotonin binding as a function of concentration. The results in Fig. 3 show that the amount of binding to the P_2 fraction was proportional to the concentration of the exogenous serotonin-^{14}C.

The endogenous serotonin content in the P_2 fraction was 0·163 μg/g of original tissue from which the P_2 fraction was prepared (iproniazid-treated animals, see Methods) and the average volume of the P_2 fraction was 0·15 ml/g (range, 0·175–0·145; 4 samples) of original tissue. Thus the concentration of the serotonin in the P_2 fraction was 1·1 μg/ml. In most experiments reported here, the concentration of serotonin in the medium was 0·9 μg/ml. Therefore, the ratio of the concentrations of exogenous

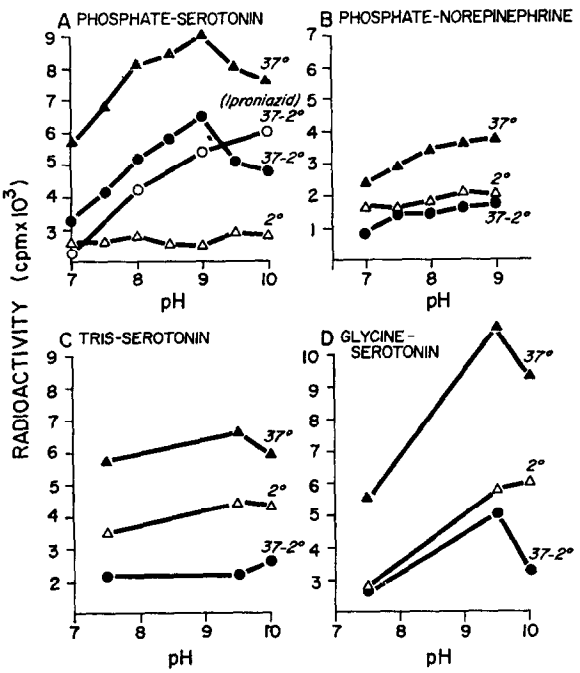

FIG. 2. Effect of pH on degree of binding of serotonin-^{14}C by the P_2 nerve-ending fraction of guinea pig brain. (A) Binding of serotonin-^{14}C, incubation in phosphate–KCl buffer. (B) Binding of norepinephrine-^{14}C, incubation in phosphate–KCl buffer. (C) Binding of serotonin-^{14}C, incubation in 0·01 M Tris–0·15 M KCl. (D) Binding of serotonin-^{14}C, incubation in 0·01 M glycine–0·15 M KCl. ○ A and all values in B indicate data from animals pretreated with iproniazid; the remaining data were obtained from experiments in which animals were not pretreated with iproniazid. ▲ 5-min incubations at 37°; △, controls at 2° (see Methods); ●, differences between values at 37° and 2°.

serotonin (added isotope) to the concentration of endogenous serotonin was 0·8. Similar computations applied to the norepinephrine binding experiments gave a ratio of 10·0.

Binding properties of subfractions of the P_2 particulate fraction and other subcellular fractions. The results in Fig. 4 show that 59 per cent of the serotonin bound to the P_2 fraction was associated with the subfraction (0·8–1·2 M sucrose) containing mainly nerve-endings.[20] The data in Table 1 indicate that of the two nerve-ending fractions (C and D) serotonin was rather specifically associated with the "cholinergic" (C) type.[7, 19]

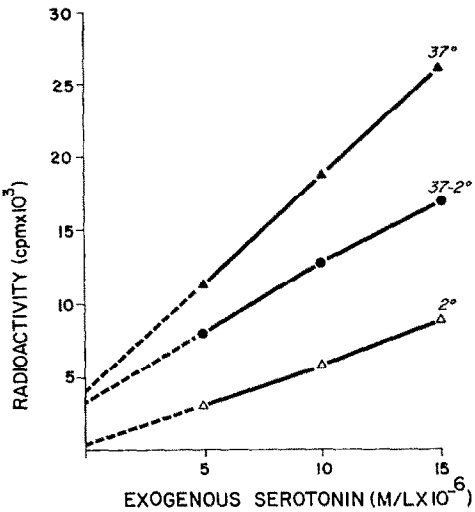

FIG. 3. Effect of concentration on degree of binding of serotonin-^{14}C by the P_2 nerve-ending fraction of guinea pig brain as a function of concentration. Incubation was for 5 min in phosphate–KCl buffer (pH 8·0). The ▲ indicate binding at 37°, the △ that at 2°, and the ● the difference.

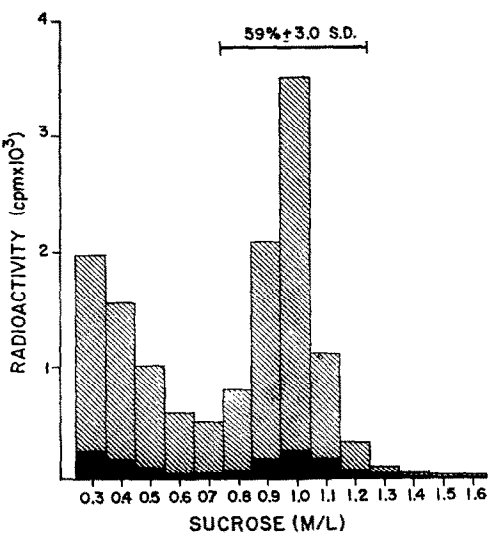

FIG. 4. Binding of serotonin-^{14}C to subcellular fractions of guinea pig brain. The P_2 nerve-ending fraction was incubated for 5 min at 37° in phosphate–KCl buffer (pH 8·0) and subfractions were then prepared according to the sucrose density method of Michaelson and Whittaker.[20] Shaded areas indicate binding at 37° as a function of sucrose concentration. Solid base areas indicate control levels at 2° (see Methods). The percentage of serotonin binding at 37° to those particles present in the region of 0·8 M to 1·2 M sucrose is indicated at the top of the figure (N = 4).

TABLE 1. SUBCELLULAR DISTRIBUTION OF BOUND SEROTONIN*

Fraction†	Ultrastructure†	Total radioactivity (%)	
		Expt. 1	Expt. 2
A‡	Myelin fragments	45	55
B‡	Membranes, synaptic debris	7	5
C	Cholinergic nerve-endings	45 } 55	37 } 45
D	Noncholinergic nerve-endings	3	3
E	Free mitochondria	<0·5	<0·5

* After incubation with serotonin-^{14}C, the nerve-ending or P_2 fraction of Whittaker *et al.*[17] was separated into its subcellular components according to the procedure of De Robertis *et al.*[19]

† According to De Robertis *et al.*[19]

‡ Complete separation of the A and B fractions was difficult, and upon removal there was some mixing.

Ruptured nerve-ending, synaptic vesicle, and microsomal fractions (prepared from equivalent amounts of brain tissue) had less capacity to bind serotonin than did the P_2 fraction (Fig. 5). Since the sum of the binding values of the synaptic vesicle and ruptured nerve-ending fractions at 37° was only 30 per cent of that of the P_2 fraction, the supernatant resulting from the preparation of the vesicle and ruptured nerve-ending fractions was further investigated by the method of equilibrium dialysis. This method failed to show any binding by the supernatant fraction. The mitochondrial fraction had little if any capacity for binding serotonin (see Fig. 4, 1·2–1·6 M sucrose, and Table 1). Fig. 6, representing composite data from several experiments, illustrates the effect on serotonin binding of treating the P_2 fractions with decreasing concentrations of sucrose.

Substrate specificity. The results in Fig. 7 show the relative binding of serotonin, histamine, norepinephrine, and tryptophan to the nerve-ending fraction.

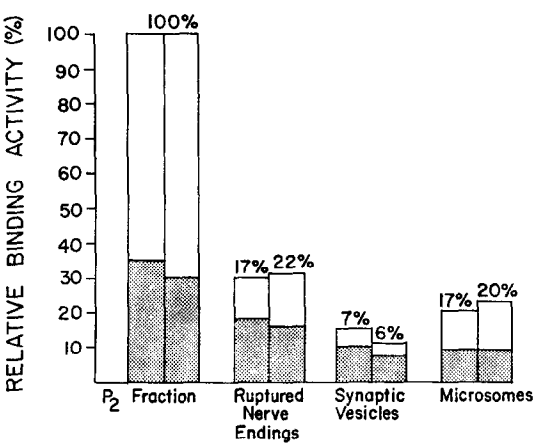

FIG. 5. Binding of exogenous serotonin-^{14}C to subcellular fractions of guinea pig brain (two experiments). The various fractions were prepared from equivalent amounts of brain and incubated for 5 min at 37° in phosphate–KCl buffer (pH 8·0). Shaded areas indicate control values at 2°. Unshaded areas show the amount of binding at 37°. Relative binding at 37° is indicated above each bar; 100 per cent has been assumed for the P_2 fraction.

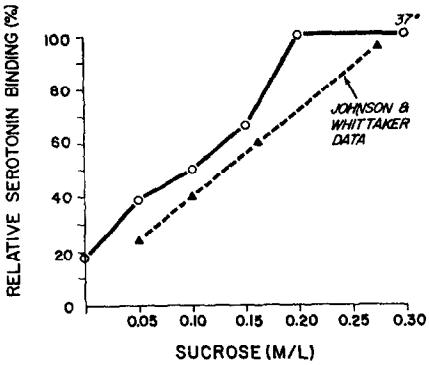

FIG. 6. Binding of serotonin-^{14}C to the P_2 nerve-ending fraction treated with different concentrations of sucrose (○). Different concentrations of sucrose were added to aliquots of the P_2 fraction and the fraction was rehomogenized and centrifuged at 11,500 *g* for 20 min to obtain the treated P_2 pellet. The pellet was resuspended in phosphate–KCl buffer (pH 8·0) and incubated at 37° for 5 min. Data were obtained from four experiments. In each, one aliquot was treated with 0·3 M sucrose, the amount of binding in this fraction was assigned the value of 100 per cent, and the relative amounts of binding by parallel aliquots treated with different concentrations of sucrose were compared with it (control values at 2° have been subtracted). ▲ represent values calculated from the data of Johnson and Whittaker[37] (see Discussion).

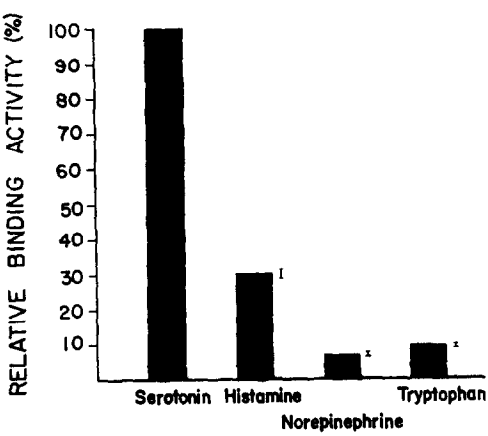

FIG. 7. Relative binding of ^{14}C-labeled histamine, norepinephrine, tryptophan, and serotonin to the nerve-ending fraction. The P_2 fraction was incubated for 5 min at 37° in phosphate–KCl buffer (pH 8·0) in the presence of equal quantities (5×10^{-6} M) of each of the compounds. The nerve-ending or B fraction was separated and the amount of bound compound determined. The amount of bound serotonin was assigned the value 100 per cent and the average relative binding of the other compounds was compared to it (control values obtained at 2° have been subtracted). Vertical bars indicate the range (N = 3 or 4).

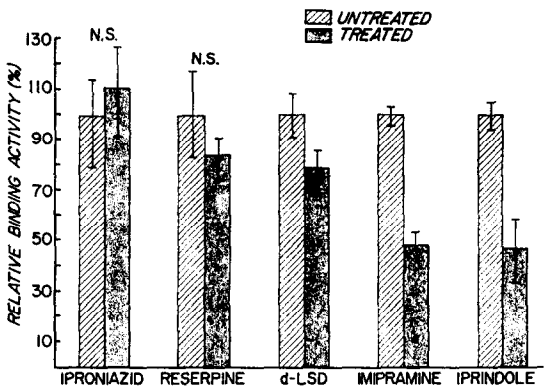

FIG. 8. Effect of drugs on binding of serotonin-^{14}C to the nerve-ending fraction. The P_2 fraction was incubated at 37° for 5 min in phosphate–KCl buffer (pH 8·0) in the presence of drugs (2×10^{-4} M final drug concentration, except reserpine which was 5×10^{-6} M). The P_2 fraction was treated in the usual manner to obtain the nerve-ending fraction (see Methods). For each experiment, four aliquots were run together, i.e. drug-treated and nondrug-treated aliquots both at 37° and 2° (control values). The binding at 2° was subtracted from that at 37° (see Methods). The average relative binding by the drug-treated sample was compared to the nondrug-treated sample which was assigned the value 100 per cent. Vertical bars indicate the range (N = 4). Data were analyzed by the *t*-test. N.S. means the test did not indicate a significant difference of the drug-treated sample from the untreated. LSD imipramine, and iprindole showed a difference at the 0·005 level of confidence.

Drug studies. The data in Fig. 8 indicate that reserpine and iproniazid did not produce a significant reduction of the binding of serotonin, but imipramine, iprindole* (Wy-3263), and LSD did reduce binding. The mean reduction of binding by imipramine was 52 per cent; by iprindole (Wy-3263), 53 per cent; and by LSD, 21 per cent.

Iproniazid and reserpine were further tested in other experiments. Reserpine was without effect in instances in which the nerve-ending fraction was prepared from animals not pretreated with iproniazid[23] and also in an experiment in which no chloride ion[24] was present in the incubation medium. When the effect of iproniazid pretreatment was examined by comparing nerve-ending fractions run in parallel from pretreated and nonpretreated animals, no apparent difference in the binding of serotonin was detected.

DISCUSSION

During the last several years, a number of investigators have reported on the binding *in vitro* of serotonin to brain fractions. For clarity of discussion, some of the data from these studies have been summarized in Table 2. It is apparent that a variety of approaches and experimental conditions were employed and that there was conflict among findings both as to the properties of the systems and the effects produced by drugs. Furthermore, several authors[4, 9, 15] described the binding as nonspecific.

* Iprindole has the following structure:

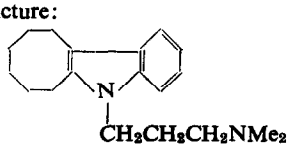

TABLE 2. COMPARATIVE DATA ON SEROTONIN BINDING TO BRAIN SUBCELLULAR FRACTIONS

Investigator(s)	Type of study, endogenous or exogenous	Source of tissue	Conc. exogenous serotonin (M)	Subcellular fractions studied	Type of binding	Medium and incubation temperature	Properties of the system	Drug effects on serotonin binding*
Giarman and Schanberg[10]	Endogenous and exogenous	Rat		Heterogenous†	"Affinity" or "absorb"	Sucrose, 0°		Reserpine ↓
Schanberg and Giarman[23]	Endogenous	Rat		Heterogenous†	"Association" or "bound"	Sucrose, 4°		Reserpine ↓ Imipramine ↑ LSD-25 ↑
Walaszek and Abood[16]	Exogenous	Rat	1×10^{-6} M	"Mitochondria"†	"Fixation"	Sucrose, 0°	Water released serotonin	Reserpine ↓ LSD-25 ↓
Whittaker[11]	Exogenous	Guinea pig	1×10^{-4}	P_2 (mitochondria and nerve-endings)	"Uptake"	Sucrose, 0°		Reserpine, N.E. ATP ↑
Gillis *et al.*[9]	Exogenous	Rat	$5{\cdot}7 \times 10^{-7}$ 2×10^{-5}	P_2 and microsomes	"Uptake, nonspecific storage"	Sucrose, 37°	P_2, not time dependent; microsomes, dep. on conc., temp., time (pH optimum, 7·2)	
Robinson *et al.*[4]	Exogenous	Rat	$2{\cdot}8 \times 10^{-6}$ 4×10^{-3}	Nerve-endings, microsomes	"Uptake, nonspecific ion-exchange"	Sucrose–glycylglycine, pH 7·4, 37°	Dep. on time, pH, temp. "nonspecific"	Iproniazid, N.E.
Haber *et al.*[15]	Exogenous	Rat	$2{\cdot}3 \times 10^{-6}$ $2{\cdot}3 \times 10^{-4}$	Whole homogenate	"Association, nonspecific"	Sucrose, 0°, 37°	Not dep. on temperature	Reserpine, N.E.
Alivisatos *et al.*[14]	Exogenous	Rat	$0\text{–}1 \times 10^{-2}$	Mitochondria, microsomes	Probably covalent	Tris-HCl, 37°	Dep. on time, particle, concn.	Iproniazid ↓
Marchbanks[12]	Exogenous	Rat	1×10^{-7} 1×10^{-2}	Nerve-endings, mitochondria	"Low, medium and high-affinity binding"	Na or K phosphate, pH 7·3, 2°	Dep. on serotonin concn.	Iproniazid ↓ LSD-25 ↓ Reserpine ↓

* The symbols ↓ and ↑ indicate decreased or increased binding of serotonin; N.E. indicates no effect.
† Probably includes mitochondria, microsomes, nerve-endings and membrane fragments.
‡ Probably includes mitochondria, nerve-endings and membrane fragments.

In our system the binding was dependent on the structure of the substrate, substrate concentration, pH, temperature, time, composition of the incubation medium, and the ionic form of the substrate. Sucrose density gradient experiments established that the binding was largely associated with the nerve-ending fraction. Anti-depressant drugs reduced the binding. In terms of the physicochemical and pharmacological criteria evaluated, the binding of serotonin was relatively specific.

While this manuscript was being prepared, Marchbanks[12] reported on a relatively specific (high affinity) type of binding of serotonin by a nerve-ending fraction. He used the inhibition of binding by known pharmacological antagonists of serotonin as a criterion to indicate a specific type of binding by nervous tissue. It may be that the type of binding reported in our work is similar to this high-affinity binding[12] because of the following similar experimental conditions and results: (1) High KCl concentrations and phosphate buffer (instead of sucrose, see below) were used in both cases. (2) Iproniazid did not reduce binding. (3) The exogenous serotonin concentration (5×10^{-6} M) was near that used by Marchbanks. (4) Both binding systems were inhibited by LSD-25. (5) The binding was associated mostly with the nerve-ending fraction.

The reason for apparent discrepancies between previous findings and those of Marchbanks[12] and our own may be explained on the basis of the properties of the incubation medium, the homogeneity of the brain fractions, and the concentration of the exogeneous serotonin (see Marchbanks[12] for a discussion of this last point). The data in Table 2 show that most investigators either used sucrose as a medium, or employed rather heterogeneous brain fractions, or both. Marchbanks[12] and we used a more homogeneous fraction, along with a salt (chloride)–phosphate buffer as a medium. Marchbanks reports that NaCl was used "to obtain a clearer determination of the high-affinity component." In exploratory experiments, we found that a sucrose medium produced nearly as much binding at 2° as at 37°. Robinson *et al.*[4] reported a similar result, i.e. their binding levels at 37° were only 1 to 1½ times the 2° level. With our chloride–phosphate medium, the binding at 37° was 3–6 times that at 2°. Binding therefore appears to depend on several physicochemical factors, and experimental conditions must be optimal in order to demonstrate the more specific type of binding.

Exploratory work indicated that the sequence of steps was also very important. The nerve-ending fraction can first be isolated by subjecting it to high concentrations of sucrose, as other investigators[4, 24] have done, or serotonin-^{14}C binding can first be allowed to occur in the P_2 fraction and the particulate subfractions can then be separated. We chose the latter method because of the possibility that high sucrose concentrations might alter the serotonin binding sites. Indeed, prolonged incubation (Fig. 1) and rupturing (Fig. 6) did reduce the binding. Once binding had occurred, we showed that the procedure used to isolate the nerve-ending fraction did not release the isotope into the medium.

With the foregoing sequence of steps, we determined the binding of serotonin to the P_2 subfractions. Since some discrepancies exist between the findings of Whittaker and De Robertis, which may in part be due to differences in methodology, we used the sucrose density methods of both groups for subfractionation of the P_2 fraction and obtained similar results with each method. The data in Fig. 4 show that about 60 per cent of the binding was associated with particles which, according to Michaelson and

Whittaker,[20] are mainly nerve-endings and contain 73 per cent of the endogenous serotonin. The remaining 40 per cent of the bound serotonin was associated with a more heterogeneous fraction containing membrane fragments, nerve-endings and a few mitochondria (2 per cent). The binding of serotonin in this heterogeneous fraction may be associated with the nerve-endings also reported to be present in this fraction.[20] Somewhat similar results were obtained in the De Robertis *et al.*[19] system which, according to the authors, separates "cholinergic" (C) from "noncholinergic" (D) nerve-endings. In our study, fractions B, C, and D contained about 50 per cent of the bound serotonin. The densities of these fractions taken together are roughly equivalent to fractions 2–5 of Michaelson and Whittaker,[20] although no exact comparison is possible. When considering only the C and D fractions of the De Robertis system, virtually all of the bound serotonin was associated with the "cholinergic" nerve-endings. Zieher and De Robertis[7] found 51 per cent of the endogenous serotonin in the C fraction and 23 per cent in the D fraction. The observation that most of the endogenously and exogenously bound serotonin is associated with "cholinergic" nerve-endings may be of interest in view of Aprison's proposal[25] that serotonin acts as a chemical modulator on the acetylcholine–cholinesterase system. However, other evidence (see Discussion in refs. 8 and 26) would favor the view that the brain contains a separate serotonergic neuronal system and thus distinct serotonergic nerve-endings which, by coincidence, are of a density close to that of the "cholinergic" nerve-endings.

Compared to the P_2 fraction, the binding in the microsomal fraction was quite small (Fig. 5). Michaelson and Whittaker[6] and Giarman and Schanberg[10] found little endogenous serotonin associated with the microsomal fraction. More significantly, in our work neither the ruptured nerve-ending nor the vesicle fraction bound much serotonin, and furthermore, the sum of the binding values of these two fractions did not approximate the binding in the P_2 fraction. At 2°, nearly 80 per cent of the binding in the P_2 fraction was accounted for in the ruptured nerve-ending and vesicle fractions, but at 37° the proportion was reduced to only about 30 per cent, which further indicates that the nature of the binding at 2° may differ from that at 37°, as the pH studies indicated (see below).

The effects of the treatment with water at 37° may be explained in two ways: (1) the spatial arrangement of the binding site was altered by the treatment; (2) a molecule containing binding sites was released into the supernatant. The equilibrium dialysis experiments with the supernatant fraction failed to indicate the presence of a nondialyzable binding molecule. However, this type of experiment does not exclude the possibility that a dialyzable binding molecule was released into the supernatant.

One might have expected more binding of serotonin in the vesicle fraction.[24] There are several possible explanations for the low binding values we obtained. (1) The vesicles may need cytoplasm or a component of the cytoplasm in order to bind serotonin. (2) The treatment with water may have altered the spatial arrangements of the binding sites. (3) The method used in preparing the vesicle fraction did not completely release the vesicles from the nerve-endings. (4) In the intact brain, relatively small amounts of serotonin are bound to the vesicles.

The data in Fig. 6 complement the foregoing water treatment experiments in that they show the effect on the binding by P_2 fractions subjected to treatment with different concentrations of sucrose. Johnson and Whittaker[27] examined the effect of different

concentrations of sucrose on the release of lactate dehydrogenase (present in the cytoplasm) from the P_2 fraction. They suggested that the P_2 fraction may contain "a mixture of nerve-ending particles of varying stability." The results taken from Table 4 of their publication have been subtracted from 100 and the resulting values plotted as solid triangles (▲) in our Fig. 6. We consider that these values give a rough indication of the percentage of intact nerve-endings. The similarity between our curve (representing the binding of exogeneous serotonin) and the curve of Johnson and Whittaker is striking.

Some evidence concerning the mechanism of serotonin binding and the specificity of this process has been presented. There appear to be at least two binding processes, since the binding was dependent on pH at 37° but not at 2° (Fig. 2A). Since the electrophoresis data showed that serotonin was in the cationic form between pH 8 and 10, a possible explanation for the pH effect at 37° is that more binding sites became available as the pH was increased. The decrease in binding between pH 9 and 10 may be due to several reasons: (1) the amount of the cationic form of serotonin decreased, as indicated by electrophoresis; (2) the binding sites were degraded by the alkaline pH; (3) the serotonin was degraded. These results suggest that one of the mechanisms regulating the concentration of serotonin in the intact brain may be local fluctuations in pH.

The ionic form of the substrate also appeared to be important, since the structurally similar tryptophan (which would be largely in the zwitterion form) was hardly bound at all (Fig. 7). In the system of Robinson *et al.*[4] the amount of the binding of serotonin and histamine was similar, and they suggested that the binding was analogous to an ion-exchange process. No such comparison is applicable to our system, since we found that norepinephrine and histamine (both cations at pH 8·0) were bound to a considerably lesser extent than serotonin.

The binding of serotonin does not appear to be an active or energy-dependent process, even though it occurred in a situation where the concentration of endogenous serotonin was greater than the exogenous serotonin-^{14}C. We found no inhibition of binding by 5×10^{-6} M parachloromercuribenzoate (PCMB). This is in agreement with the work of Schanberg[5] and of Robinson *et al.*[4] who also found no effect by 1×10^{-4} M PCMB.

The antidepressants, imipramine and iprindole (Wy-3263), caused the most striking reduction of serotonin binding. Imipramine inhibition of serotonin binding has been demonstrted in peripheral nerve tissues,[3, 28] but it appears that this effect has not been reported for the central nervous system. Recently, we have determined the effect of imipramine on the binding of serotonin in the intact brain by the intraventricular injection method of Glowinski and Axelrod.[29] Preliminary results showed a reduction of serotonin-^{14}C both in the whole brain and in the P_2 fraction.

Our results with imipramine appear to be at variance with those reported by Kivalo *et al.*[30] and by Schanberg and Giarman,[23] who found that serotonin brain levels were elevated after imipramine treatment and that imipramine increases the amount of "particle"-bound endogenous serotonin. Several reasons may possibly explain these differences. (1) Schanberg and Giarman were measuring total serotonin binding to a very heterogeneous particulate fraction and not to nerve-endings. (2) The exogenous binding sites may be different form the endogenous sites. (3) Different species and procedures were used.

Our data and those of Marchbanks[12, 24] and of Walaszek and Abood[16] show that LSD inhibited the binding of serotonin to nerve-endings. LSD could be acting either as a weak blocker of the binding of serotonin by the nerve-endings or acting in its postulated capacity as an antagonist to serotonin by inhibiting binding to post-synaptic membranes, which are apparently present in the nerve-ending fraction.[31]

In our system, reserpine produced no significant reduction of the binding of serotonin under the conditions tested (i.e. in the presence or absence of either iproniazid[23] or chloride ion[24]). Whittaker[11] and Haber *et al.*[15] also observed no reserpine effect, but Marchbanks[12] reported that it reduced the binding. Most of the evidence in the literature[32, 33] favors the view that reserpine acts mainly at the level of the storage granule. Marchbanks used very long incubation periods (12–48 hr.), which would allow time for reserpine to penetrate into the nerve-ending and affect the binding of serotonin to synaptic vesicles. The short incubation periods employed in the present work may not have been long enough for reserpine to effect such a reduction.

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