# Antibodies to Morphine, Barbiturates, and Serotonin

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ADVANCES in any discipline usually are tied intimately to technological advances. Berson and Yalow (5a) have made available to the endocrinologist, physiologist, biochemist, and pharmacologist a very sensitive and specific method for the quantitative measurement of hormones, enzymes, and drugs in biological fluids by developing the radioimmunoassay technique. The localization and identity of these biologically active substances has also been facilitated greatly by such immunological procedures as immunofluorescence, globulin labeled with an electron-dense marker for electron microscopy, and immunodiffusion in gel.

Small molecules which are pharmacologically active but are not antigenic may be rendered antigenic by chemically coupling them to a macromolecular substance, usually a protein or polysaccharide. The antibody produced may be capable of binding, or neutralizing the biological activity of the uncombined small molecule. Moreover, utilizing this antibody, an immunoassay may be devised for a low molecular weight compound.

The application of these techniques to the opiate alkaloids permits the testing of the hypothesis proposed by Cochin (8) that the tolerance to morphine may be an immunological phenomenon. To study this problem, three morphine derivatives were conjugated to a protein to elicit antibodies which might be capable of modifying the pharmacological action of morphine.

Three morphine-protein derivatives were

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prepared by coupling the morphine derivatives to bovine serum albumin. The low molecular weight molecule in these conjugates was linked to the protein either by an amide bond or by coupling through a diazo bond *via* the amino group on the aromatic protein of the hapten. Figure 1 shows the structural formulae of the three derivatives that were used for conjugation. The compound 3-O-carboxymethylmorphine was prepared by reaction of the free base with sodium-β-chloroacetic acid in absolute methanol (4, 18). The 6-morphenyl hemisuccinate was prepared by the reaction of morphine with succinic anhydride in pyridine and 2-(p-amino-phenylazo) morphine was prepared by diazotizing p-aminoacetanilide to morphine and then the free amine in the p-position was formed by either hydrolysis or saponification.

The three morphine-protein conjugates described above were antigenic when New Zealand albino rabbits were given several injections of 1 mg of each complex dissolved in a phosphate-buffered saline pH 7.4 and emulsified with an equal volume of Freund's adjuvant.

The specificity of the antibodies generated by the three morphine immunogens was determined by incubating various opiate alkaloids or their surrogates with the antibody in the presence of labeled dihydromorphine and measuring the inhibition of the antibody-dihydromorphine $^3H$ complex formation (tables 1 and 2). The congeners of morphine, codeine, and heroin all bind to the antibodies produced by the three immunogens and cause a 50 % inhibition of the antibody isotope complex at about the same concentration as does morphine. Antibodies stimulated by the 3-O-carboxymethylmor-
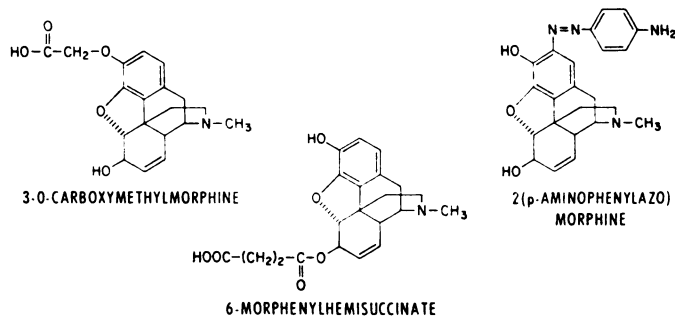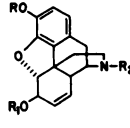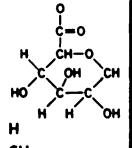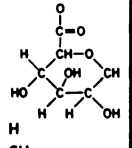
FIG. 1. Morphine derivatives used for preparation of morphine-protein conjugates.

TABLE 1
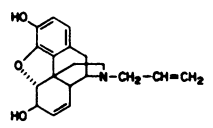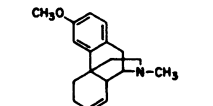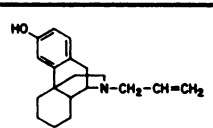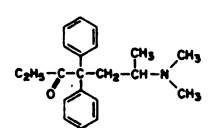
Competition with $^3H$-dihydromorphine for binding sites*
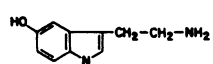
| STRUCTURE | COMPOUND | CONCENTRATION WHICH INHIBITS LABELED Ag-Ab COMPLEX 50% | | |
|---|---|---|---|---|
| | | 3-0-CARBOXYMETHYL-MORPHINE | 6-MORPHENYLHEMI-SUCCINATE | 2(p-AMINOPHENYLAZO)-MORPHINE |
| MORPHINE (m.w. 285) | R = H<br>R₁ = H<br>R₂ = CH₃ | 0.50 ng | 0.50 ng | 0.75 ng |
| CODEINE (m.w. 299) | R = CH₃<br>R₁ = H<br>R₂ = CH₃ | 0.50 ng | 0.50 ng | 0.90 ng |
| HEROIN (m.w. 369) | R = CH₃-CO<br>R₁ = CH₃-CO<br>R₂ = CH₃ | 0.50 ng | 0.50 ng | 2.30 ng |
| NORMORPHINE (m.w. 271) | R = H<br>R₁ = H<br>R₂ = H | 0.50 ng | 7.0 ng | 60.0 ng |
| MORPHINE-3-MONOGLUCURONIDE | R =<br>R₁ = H<br>R₂ = CH₃ | 200.0 ng | 2.0 ng | 40.0 ng |

* Experimental conditions are described in detail elsewhere (31).

phine-bovine serum albumin conjugate produced a 50 % inhibition of the isotope complex with normorphine at the same concentration as morphine. The antibodies elicited by the 6-morphenylhemisuccinate-bovine serum albumin also recognized normorphine almost as well as morphine. However, antibodies stimulated by 2-(p-amino-phenylazo) morphine-bovine serum albumin required much higher concentrations to produce 50% inhibition. The insertion of a large hydrophilic group such as a glucuronide at the 3-OH group markedly diminishes the binding by antibodies stimulated by the 3-O-carboxymethylmorphine conjugate or the 2-(p-aminophenylazo) morphine-protein immunogen. However, the antibodies stimulated by the 6-morphenyl hemisuccinate im-

TABLE 2

*Competition with $^3H$-dihydromorphine for binding sites*

| COMPOUND | STRUCTURE | CONCENTRATION WHICH INHIBITS LABELED Ag-Ab COMPLEX 50% | | |
|---|---|---|---|---|
| | | 3-0-CARBOXYMETHYL-MORPHINE | 6-MORPHENYLHEMI-SUCCINATE | 2(p-AMINOPHENYLAZO)-MORPHINE |
| NALORPHINE (m.w. 311) | | 200.0 ng | 500.0 ng | 15.0 ng |
| DEXTRO-METHORPHAN (m.w. 271) | | 0% at 200.0 ng | 25% at 200.0 ng | 25% at 200.0 ng |
| LEVALLORPHAN (m.w. 283) | | 0% at 200.0 ng | 20% at 200.0 ng | 200.0 ng |
| METHADONE (m.w. 346) | | 200.0 ng | 0% at 200.0 ng | 0% at 200.0 ng |
| SEROTONIN (m.w. 176) | | 0% at 200.0 ng | 0% at 200.0 ng | 0% at 200.0 ng |

munogen bound morphine glucuronide at a concentration slightly greater than morphine although on a molar basis morphine glucuronide was almost as effective as morphine. The effect of synthetic surrogates of morphine was also studied (table 2). Immunizing with either carboxymethylmorphine or the 6-morphenyl hemisuccinate group generated antibodies which failed to bind nalorphine until high concentrations were achieved, so that the allyl group on the nitrogen appears to be an important determinant group. The inhibitory capacity of nalorphine was more effective in producing 50% inhibition of precipitation of labeled hapten-protein complex with antibodies elicited by the azo derivative of morphine. The synthetic morphinans, dextromethorphan and levallorphan, were not recognized to any extent by any of the three antibodies. Thus modifying the furan oxygen ring apparently abolishes a determinant group which the antibody can recognize. Methadone, which lacks the classical phenanthrene ring structure of morphine also required very high concentrations in relation to morphine to elicit a 50% inhibition of the antibody-hapten complex. Recently, there has been speculation on the interrelation between morphine tolerance and dependence and the biogenic amine serotonin (33, 34). Inhibition of serotonin synthesis was reported to produce a loss of tolerance and dependency to morphine (23), and it was thus of interest to ascertain whether the antibodies recognized serotonin. The antibodies failed to bind serotonin at the doses tested.

A vast amount of work has been done to show that antibodies can inhibit the action of enzymes (7), and it also has been demonstrated that the biological activity of various hormones such as insulin (24), growth

hormone (16), thyrotropic activity (34), chorionic gonadotropin (30), adrenocorticotrophic hormone (ACTH) (32), and steroids (15, 22) can be inhibited by antibodies. It has also been shown that drug toxicity can be modified by the development of antibodies against the haptenic drug (6, 27, 28). The question was then posed whether antibodies directed against the morphine alkaloid could modify the pharmacology of morphine. As the principal therapeutic application of the narcotic is for the relief of pain, studies were done to ascertain whether the antibodies could modify the analgesia produced by morphine. Pain was elicited in mice by the administration of a chemical irritant p-phenylquinone, and which manifested itself by a characteristic abdominal stretch response which could be quantitated by counting the numbers of movements in a 5-min period. Mice were immunized for either 6 or 16 weeks and then tested to ascertain the presence of antibodies. In table 3 it is shown that the analgesia produced by morphine was dose-dependent, and those mice which were immunized actively had more movements than did the control mice. Studies are currently in progress to ascertain to what extent brain concentrations of the opiate alkaloid have been reduced by the antibodies. What has been shown is that the distribution of labeled dihydromorphine has been altered markedly in mice immunized by the 3-O-carboxymethylmorphine immunogen (5).

The problems of tolerance and habituation also occur with the barbiturates, an important class of central nervous depressants. These drugs which are used to produce depression ranging from mild sedation to surgical anesthesia are one of the most widely prescribed group of drugs. Since these compounds have such a widespread use, both in therapeutics and as a class of drugs being abused, it is critical that methods be available which are both sensitive, specific, and quick to perform. One of the most widely employed methods currently available for the quantitative analysis of barbiturates

TABLE 3

Analgesic effect of morphine in immunized mice

| Treatment | Analgesia,*† Morphine (mg/kg) | | | |
|---|---|---|---|---|
| | 0 | 0.5 | 0.75 | 1.0 |
| Experiment 1‡ | | | | |
| Control | 100% | 72% | 13% | 9%* |
| Morphine immunogen | 100% | 74% | 96% | 42% |
| Experiment 2‡ | | | | |
| Control | 100% | 25% | 18% | |
| Morphine immunogen | 100% | 93% | 76% | |

* Analgesia was estimated by the para-phenylquinone (PPQ) abdominal stretch test. The average movements at each dose of morphine is expressed as a percent (%) of a group of saline-treated controls which received only PPQ. In experiment 1, morphine sulfate and in experiment 2, morphine hydrochloride was injected s.c., in the doses indicated, 15 min before PPQ and movements counted 10 min after PPQ for 5 min.

† The observer of analgesic tests did not know prior treatment of mice.

‡ In experiment 1 weanling mice received s.c. 0.1 ml of a 50% emulsion of complete Freund's adjuvant (control) or 1 μg of morphine immunogen in 50% complete Freund's adjuvant (morphine immunogen) twice the first week and every 7 to 10 days thereafter for 6 weeks. Each dose of morphine was given to each of 5 mice and compared to responses observed in 5 control mice which received no morphine.

In experiment 2, mice were injected in a similar fashion for 16 to 20 weeks with one exception. From the 3rd to 10th week, saline was substituted for the Freund's adjuvant in the control group and the morphine-immunogen-plus-adjuvant group. When these mice were examined for effects of morphine during the 10th week, no difference occurred among the groups. Adjuvant was then reinstated in the adjuvant group and morphine-immunogen-plus-adjuvant group for each subsequent weekly injection. Tests were done 4 days after the last injection in 10 mice from each group, 5 for each of the two doses of morphine, and compared to 5 control mice which received no morphine.

requires solvent extraction (9, 20, 21), and in some instances filtration and evaporation. Therefore it was felt that a radioimmunoassay would be most advantageous. The barbiturate hapten, 5-allyl-5-(β-carboxyl-α-

methyl-ethyl) barbituric acid was conjugated to the protein bovine γ-globulin. The barbiturate was converted to 5-allyl-5-(1-p-nitrophenyloxycarbonylisopropyl) barbituric acid by reacting the base with p-nitrophenol in dimethylformamide to form an ester of the barbituric acid. A nucleophilic attack on the ester by the amine group of the protein displaces the p-nitrophenol and replaces it with an amide linkage (fig. 2).
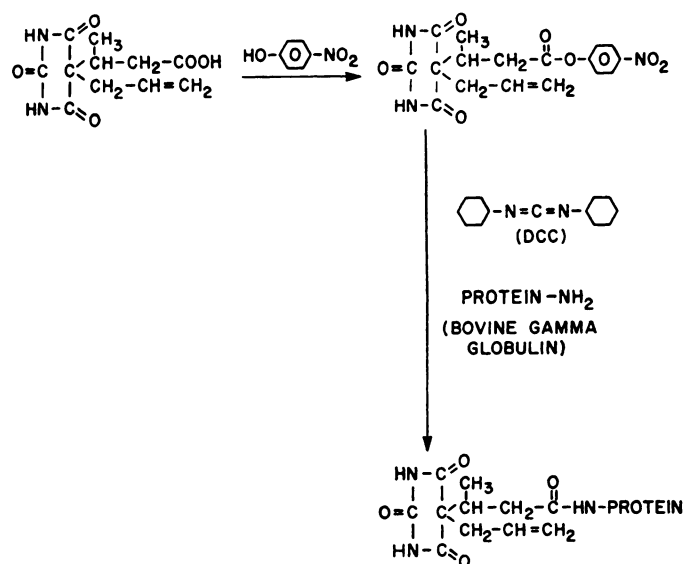
The specificity of the antibodies was de-



Fig. 2. Experimental conditions are described in detail elsewhere (14).

TABLE 4

Competition with barbital-$^{14}C$ for binding sites



| Compound | $R_1$ | $R_5$ | $R'_5$ | Dose which inhibits labeled barbital-$C^{14}$-Ab complex 50% |
|---|---|---|---|---|
| 1) Barbital | -H | -$C_2H_5$ | -$C_2H_5$ | 20 ng |
| 2) Pentobarbital | -H | -$C_2H_5$ | -CH(CH_3)-CH_2-CH_2-CH_3 | 10 ng |
| 3) Phenobarbital | -H | -$C_2H_5$ | -⬡ | 10 ng |
| 4) Secobarbital | -H | -CH_2-CH=CH_2 | -CH(CH_3)-CH_2-CH_2-CH_3 | 5 ng |
| 5) Metharbital | -CH_3 | -$C_2H_5$ | -$C_2H_5$ | >500 ng |
| 6) Mephobarbital | -CH_3 | -$C_2H_5$ | -⬡ | >500 ng |
| 7) Hexobarbital | -CH_3 | -CH_3 | -⬡ | >500 ng |

termined by incubating the compound to be tested with the antiserum in the presence of labeled barbital-$^{14}$C and measuring the inhibition of the antibody-barbital-$^{14}$C complex formation. The antibody has a high affinity for barbital, pentobarbital, phenobarbital, and secobarbital; these compounds differ only by their substituents on carbon 5. However, if a methyl substituent replaces the

hydrogen of the ring nitrogen, as is the case with metharbital and mephobarbital which are identical to barbital and phenobarbital except for the presence of the methyl group, then the antibody fails to bind these compounds. Similarly, hexobarbital was not recognized by the antibody. Substitution on carbon 5 also is critical, as the antibody fails to bind barbituric acid (table 4). Table 5

TABLE 5

*Competition with barbital-$^{14}$C for binding sites*



| Compound | $R_1$ | $R_5$ | $R'_5$ | Dose which inhibits labeled barbital—$C^{14}$—Ab complex 50% |
|---|---|---|---|---|
| 1) Thiopental | —H | —$C_2H_5$ | $\overset{\displaystyle CH_3}{\overset{\displaystyle |}{—CH—CH_2—CH_2—CH_3}}$ | 100 ng |
| 2) 2—Thiobarbituric acid | —H | —H | —H | >500 ng |

TABLE 6

*Competition with barbital-$^{14}$C for binding sites*

| Compound | Structure | Dose which inhibits labeled barbital—$C^{14}$—Ab complex 50% |
|---|---|---|
| 1) Diphenylhydantoin |  | >500 ng |
| 2) 2-Thiohydantoin |  | >500 ng |
| 3) 5,5 Diphenyl-2-thiohydantoin |  | >500 ng |
| 4) 2-Imidazolidinethionine |  | >500 ng |

indicates that the sulfur analogue is not recognized by the antibody as effectively as the oxy-analogue. Substituting a 5-membered ring in which the urea portion of the molecule is maintained, as in the case of hydantoin derivatives, results in a failure to be recognized by the antibody (table 6). Studies were performed also to ascertain the ability of the barbiturate-directed antibodies to bind a number of endogenously occurring 6-membered pyrimidines. The antibodies failed to bind either thymine or uracil both of which lack the carbonyl group at carbon 6 as well as the double substitution on carbon 5 (table 7). Similarly cytosine and 5-methylcytosine were not recognized by the antibody; both of these compounds, al-

though they have a urea portion similar to the barbiturates, have an amino group at carbon 4 as well as a 5-methyl group. In addition, it was found that urea alone was not bound by antibody. It is also interesting that glutethimide is not recognized although it is similar in structure to phenobarbital except it lacks the ring nitrogen at carbon 3 and the carbonyl group at carbon 4. Caffeine also failed to inhibit the antibody-barbital-$^{14}$C complex formation.

The radioimmunoassay was applied to determine plasma barbiturate concentrations after intraperitoneal administration of either pentobarbital or barbital (fig. 3). These barbiturates were selected because they have different durations of pharma-

## TABLE 7
### Competition with barbital-$^{14}$C for binding sites

| Compound | Structure | Dose which inhibits labeled barbital-$C^{14}$-Ab complex 50% |
|---|---|---|
| 1) Thymine | HN—CH<br>\| \|\|<br>O=C  C—CH$_3$<br>\| \|<br>HN—C=O | >500 ng |
| 2) Uracil | HN—CH<br>\| \|\|<br>O=C  CH<br>\| \|<br>HN—C=O | >500 ng |
| 3) Cytosine | HN—CH<br>\| \|\|<br>O=C  CH<br>\| \|<br>N=C—NH$_2$ | >500 ng |
| 4) 5-Methylcytosine | HN—CH<br>\| \|\|<br>O=C  C—CH$_3$<br>\| \|<br>N=C—NH$_2$ | >500 ng |
| 5) Urea | NH$_2$<br>\|<br>O=C<br>\|<br>NH$_2$ | >1000 ng |
| 6) Glutethimide | HN—C=O<br>\| \|<br>O=C  C<(O)$_{C_2H_5}$<br>\| \|<br>H$_2$C—CH$_2$ | >500 ng |

FIG. 3. Plasma barbiturate concentrations determined by radioimmunoassay.

cological action. Pentobarbital had a $T_{1/2}$ of 1.7 hr whereas barbital had a $T_{1/2}$ of 19.3 hr.

The biogenic amine, serotonin, has been suggested as having a physiological role and is involved in some human diseases (25). An association has also been made between serotonin and emotional stability (1, 10). Although a great number of methods for measuring serotonin in biological fluids have been described, it would be an improvement to have a method which can measure the amine with rapidity as well as with greater sensitivity and specificity in various biological fluids. Radioimmunoassay offers such a potential.

Antibodies specific for serotonin have been described by several authors (13, 17, 26). However, the titer of these antibodies seems to have been generally low and they have never been used for a radioimmunoassay. The protein has been conjugated to 5-hydroxytryptamine through the amine on the side chain or by the nitrogen of the indole nucleus. Another site amenable for conjugation is the aromatic ring, and thus *para*-

aminophenylalanine was coupled to bovine serum albumin with a carbodiimide reagent. The *para*-amino group was then diazotized so that an electrophilic attack on the serotonin molecule occurred. The method of synthesis and the presumed structure of the immunogen is illustrated in figure 4. Rabbits immunized with the immunogen, which was dissolved in phosphate-buffered saline pH 7.4 and emulsified with an equal volume of complete Freund's adjuvant, for several weeks were then bled from the central ear artery. In a few cases, the blood was allowed to clot at room temperature for 1 hr, stored at 4°C overnight and then the serum was separated by centrifugation at 10,000 rpm for 30 min. Most bleedings, however, were collections of blood mixed with 250 U of heparin to yield a final concentration of 1-20 U/ml. The blood was cooled immediately and centrifuged at 1000 rpm for 15 min. The supernatant was then transferred to new tubes and the plasma separated from thrombocytes by centrifugation. The latter steps ensured minimal interference from endogenous rabbit blood serotonin.
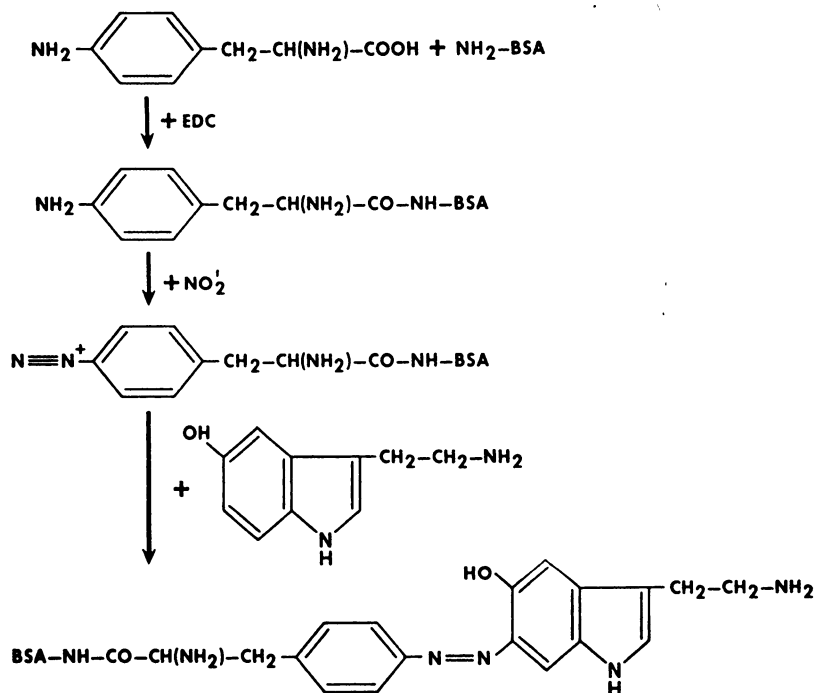
Fig. 4. Method of synthesis and presumed structure of immunogen.

Various dilutions of serum or plasma from the immunized rabbits were incubated with approximately 2000 cpm of $^3$H-serotonin creatinine sulfate in the dark at 4°C overnight. After incubation, the antibody-bound serotonin was separated with the Farr procedure (12). The antibody-serotonin complex was then dissolved in 0.5 ml of NCS solubilizer and the radioactivity was counted in a liquid scintillation spectrometer. Binding of $^3$H-serotonin by normal rabbit serum or plasma was just slightly above background. However, sera from immunized rabbits, even at a final dilution of 1:5, never bound more than 40% of the added radioactivity. On the other hand, thrombocyte-free plasma from the same animals was more than 10 times as effective, a final dilution of 1:50 binding about 50%. The diminished binding of added serotonin obtained with antiserum instead of plasma was thought to be caused by the normally occurring serotonin released into the serum from the thrombocytes during the clotting process. It is known that the serotonin content of

rabbit thrombocytes and rabbit serum is very high (11, 19), and antibody molecules isolated from such serum would be expected to have their binding sites occupied by serotonin and, therefore, exhibit a lower affinity for added antigen. In an attempt to prove this point, 5 ml of antiserum was dialyzed against 250 ml of 6 M urea for 10 hr and thereafter against 3 daily changes of 2 liters of buffered saline for 1 week. The binding of $^3$H-serotonin by antiserum treated by this procedure was in fact increased very much and approached the values seen with plasma.

All the following experiments were performed with thromobocyte-free plasma instead of serum from the immunized rabbits. The addition of increasing amounts of unlabeled serotonin to a constant amount of $^3$H-serotonin and antibody resulted in competitive inhibition of the formation of $^3$H-serotonin-antibody complex. By comparing this inhibition with the binding of $^3$H serotonin by antibody in the absence of unlabeled serotonin a standard curve could be

generated. As shown in figure 5, less than 1 ng of serotonin can be detected by this method and 6.5 ng produced a 50% inhibition of bindings. Several analogues of serotonin, at various concentrations, were tested to determine the specificity of the radioimmunoassay (fig. 6). The antibodies seem to be specific both for the ring structure and especially for the side chain of the serotonin molecule. The antibody fails to bind those 5-hydroxyindole analogues in which a substitution is made on the side chain as seen in the cases of 5-hydroxytryptophan and N-acetylserotonin. However, 5-methoxytryptamine is almost as effective as serotonin in being bound by the antibody
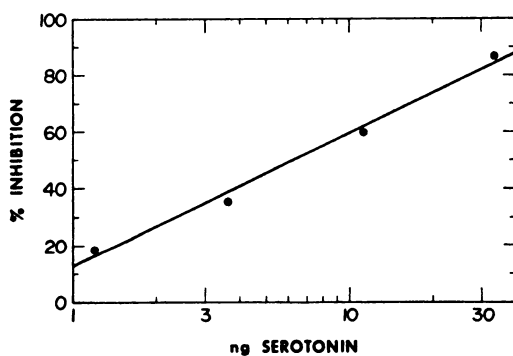


Fig. 5.

while tryptamine is about 26 times weaker. Binding to the antibody also decreases considerably in the absence of the side chain, as in 5-hydroxyindole. An important aspect of this assay is that 5-hydroxyindoleacetic acid, the major metabolite of serotonin, does not interfere with the assay at all, even when 1000 ng are added.

Although 5-methoxytryptamine is recognized by the antibody, levels of this metabolite can be expected to be low in biological fluids so that it would not interfere with the radioimmunoassay for serotonin. The enzyme 5-hydroxyindole-O-methyltransferase is located exclusively in the pineal gland (2). Also, N-acetylserotonin is a much better substrate for this enzyme than serotonin itself (3). Tryptamine, however, occurs normally in human urine (29). Although normal levels are too low to interfere with the serotonin radioimmunoassay, tryptamine levels rise after inhibition of the enzyme monoamine oxidase (29). Under such conditions, the value of the described radioimmunoassay might be limited.

For the pharmacologist, methods are critically needed for estimating the circulating levels of drugs. Most drugs have chemical characteristics which permit their
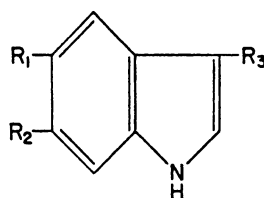


| | R1 | R2 | R3 | 50% INHIBITION |
|---|---|---|---|---|
| SEROTONIN | -OH | -H | -CH2-CH2-NH2 | 6.5 ng |
| 5-METHOXYTRYPTAMINE | -OCH3 | -H | -CH2-CH2-NH2 | 7.0 ng |
| TRYPTAMINE | -H | -H | -CH2-CH2-NH2 | 170 ng |
| 5-HYDROXYTRYPTOPHANE | -OH | -H | -CH2-CH(NH2)-COOH | >1000 ng (81%) |
| 5-METHOXYTRYPTOPHANE | -OCH3 | -H | -CH2-CH(NH2)-COOH | >1000 ng (83%) |
| 5-HYDROXYINDOLE | -OH | -H | -H | >1000 ng (87%) |
| N,N-DIMETHYLTRYPTAMINE | -H | -H | -CH2-CH2-N(CH3)2 | >1000 ng (90%) |
| N-ACETYLSEROTONIN · | -OH | -H | -CH2-CH2-NH-CO-CH3 | >1000 ng |
| MELATONIN | -OCH3 | -H | -CH2-CH2-NH-CO-CH3 | >1000 ng |
| 5-HYDROXYINDOLACETIC ACID | -OH | -H | -CH2-COOH | >1000 ng |
| 5,6-DIHYDROXYTRYPTOPHANE | -OH | -OH | -CH2-CH(NH2)-COOH | >1000 ng |
| TRYPTOPHANE | -H | -H | -CH2-CH(NH2)-COOH | >1000 ng |
| TYRAMINE | | | | >1000 ng |
| TYROSINE | | | | >1000 ng |

Fig. 6. Serotonin analogues used to determine specificity of radioimmunoassay.