variations in heart rate make these events prime candidates for clinical monitoring. Since the detection scheme employed here depended on temporal spread factors, it is tempting to believe that small, subtle alterations in ventricular physiology might be detected from real time ECG analyses. Such detection should find exceptionally wide clinical and experimental usefulness. Finally, the concept of temporal spread might also prove useful for the analysis of other portions of the ECG.

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References and Notes

1. W. Klaus, G. Kurchinsky, H. Lullman, *Arch. Exp. Pathol. Pharmakol.* **242**, 480 (1962); A. Grossman and R. F. Furchgott, *J. Pharmacol. Exp. Ther.* **145**, 162 (1964); K. S. Lee, D. H. Yu, D. I. Lee, R. Burnstein, *ibid.* **132**, 139 (1962); M. Vasselle, J. Karis, B. F. Hoffman, *Amer. J. Physiol.* **203**, 433 (1962); M. Vasselle, K. Greenspan, B. F. Hoffman, *Circ. Res.* **13**, 132 (1963); E. Krueger and K. Unna, *J. Pharmacol. Exp. Ther.* **76**, 282 (1942).
2. D. H. Singer and R. E. Ten Eick, *Progr. Cardio. Dis.* **11**, 488 (1969); Y. Watanabe and L. S. Driefus, *Amer. Heart J.* **76**, 114 (1968).
3. C. L. Feldman, P. G. Amazeen, M. D. Klein, B. Lown, *Comp. Biomed. Res.* **3**, 666 (1970); J. Haywood, V. K. Murthy, G. A. Harvey, A. Saltzberg *ibid.*, p. 15; G. C. Oliver, F. M. Nolle, G. A. Wolff, J. R. Cox, H. D. Ambos, *ibid.* **4**, 523 (1971); J. S. Geddes and H. R. Warner, *ibid.*, p. 493.
4. G. K. Moe and J. Han, in *Digitalis*, C. Fisch and B. Surawiecz, Eds. (Grune & Stratton, New York, 1969), p. 110.
5. In some studies in which very high doses (20 to 30 mg/kg) were used, complete heart block occurred within 5 minutes of ouabain injection. Prior treatment with atropine eliminated vagal influences, and spreading responses were revealed, as discussed above, with the highest doses used (30 mg/kg).
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Serotonin: Radioimmunoassay

Abstract. *The development of a radioimmunoassay for serotonin is described. Serotonin is made antigenic by coupling it to bovine serum albumin. Thrombocyte-free plasma instead of serum from the immunized animals is used for radioimmunoassay. Less than 1 ng serotonin can be measured by this procedure.*

The biogenic amine serotonin has been implicated as having a physiological role and has been suggested as being involved in many human diseases (*1*). An association has also been made between serotonin and emotional stability (*2*). To evaluate the role of serotonin it is critical to have a method that can measure the amine with great sensitivity and specificity in various biological fluids. Radioimmunoassay offers such a potential.

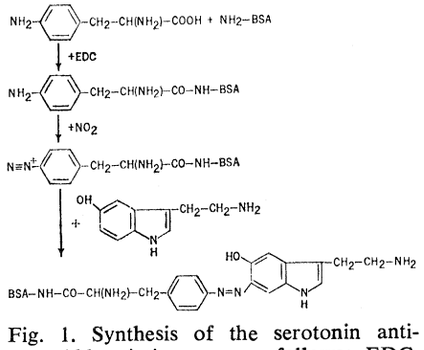

Fig. 1. Synthesis of the serotonin antigen. Abbreviations are as follows: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; BSA, bovine serum albumin.

Antibodies specific for serotonin have been described by others (*3*). However, these antibodies seem to have had generally low titers, and they have never been used for a radioimmunoassay. A number of methods for the determination of serotonin in biological fluids and tissues have been described (*4*). In this report we describe the production of antibodies against serotonin in rabbits and their use in a specific and sensitive radioimmunoassay.

To 50 mg of DL-*p*-aminophenylalanine, dissolved in 5 ml of distilled water, 50 mg of bovine serum albumin (BSA) and 50 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (*5*) were added. The mixture was incubated at room temperature overnight and then dialyzed for 5 days against distilled water with three changes (2 liters each) per day. Then the *p*H was adjusted to 1.5 with 1*N* HCl, and the following procedure was performed at 0° to 4°C. One hundred milligrams of $NaNO_2$, dissolved in 1 ml of distilled water, was added slowly dropwise, followed by 50 mg of ammonium sulfamate in 1 ml of distilled water. The diazotized protein solution was then added dropwise to 100 mg of serotonin creatinine sulfate, dissolved in 10 ml of 0.1*M* borate buffer, *p*H 9.0, with constant stirring. The *p*H was maintained above 8.0 by the addition of borate buffer. A dark red color developed almost immediately. The preparation was stirred overnight in the dark at 4°C and then dialyzed exhaustively against distilled water. For determination of the amount of serotonin coupled to the protein carrier 1 μc of [^{3}H]serotonin (*6*) was added with unlabeled serotonin, and the above procedure was followed. By measuring the radioactivity of the dialyzates and the antigen solution, it was calculated that 5.7 percent of the serotonin had been coupled, corresponding to about 20 moles of serotonin per mole of BSA (molecular weight, 70,000). Figure 1 illustrates the method of synthesis and the presumed structure of the immunogen.

Rabbits were immunized with 150 μg of the immunogen which had been dissolved in 0.5 ml of phosphate-buffered saline, *p*H 7.4, and emulsified with an equal volume of complete Freund's adjuvant; 0.25 ml was injected into each foot pad. Booster injections were given weekly for 2 weeks, and thereafter every 6 to 8 weeks. Bleedings were taken from the central ear artery, the first one as a control before immunization, and then 1 to 2 weeks after booster injections. In a few cases, the blood was allowed to clot at room temperature for 1 hour and at 4°C overnight, and then the serum was separated by centrifugation at 10,000 rev/min for 30 minutes. Most bleedings, however, were taken into 250 units of

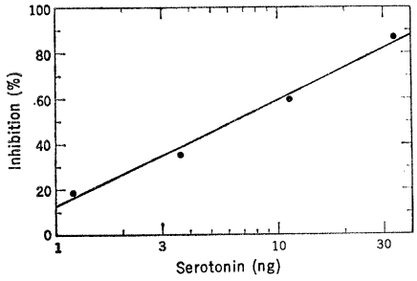

Fig. 2. Inhibition by nonradioactive serotonin of binding of [^{3}H]serotonin by antibody to a serotonin–bovine serum albumin complex (final dilution 1 : 50). The [^{3}H]-serotonin content of the assay mixture was approximately 2000 count/min. In the absence of nonradioactive serotonin, the antibody can bind 50 percent of the radioactivity.

heparin to a final concentration of 1 to 20 unit/ml. The blood was cooled immediately and centrifuged at 1000 rev/min for 15 minutes. Then the supernatant was tranferred to new tubes, and the plasma was separated from thrombocytes by centrifugation at 7000 rev/min for 30 minutes.

Various dilutions of serum or plasma from the immunized rabbits were incubated with approximately 2000 count/min of [^{3}H]serotonin creatinine sulfate (specific activity, 7.3 c/mmole) (*6*) in the dark at 4°C overnight. After incubation, saturated ammonium sulfate in a volume equal to the incubation mixture (0.5 ml) was added to all tubes. The precipitate containing the antibody-bound serotonin was washed twice with 50 percent saturated ammonium sulfate and dissolved in 0.5 ml of NCS solubilizer (Amersham/Searle); the radioactivity was then counted in a liquid scintillation spectrometer. Binding of [^{3}H]serotonin by normal rabbit serum or plasma was just slightly above background. However, serums from immunized rabbits, even at a final dilution of 1 : 5, never bound more than 40 percent of the added radioactivity. On the other hand, thrombocyte-free plasma from the same animals was more than ten times more effective, a final dilution of 1 : 50 binding about 50 percent. 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 (*7*). 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 hours and thereafter against three 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 thrombocyte-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 Fig. 2, less than 1 ng of serotonin can be detected by this method and 6.5 ng produced a 50 percent inhibition of binding.

	R_1	R_2	R_3	50% INHIBITION
SEROTONIN	$-OH$	$-H$	$-CH_2-CH_2-NH_2$	6.5 ng
5-METHOXYTRYPTAMINE	$-OCH_3$	$-H$	$-CH_2-CH_2-NH_2$	7.0 ng
TRYPTAMINE	$-H$	$-H$	$-CH_2-CH_2-NH_2$	170 ng
5-HYDROXYTRYPTOPHAN	$-OH$	$-H$	$-CH_2-CH(NH_2)-COOH$	>1000 ng (81%)
5-METHOXYTRYPTOPHAN	$-OCH_3$	$-H$	$-CH_2-CH(NH_2)-COOH$	>1000 ng (83%)
5-HYDROXYINDOLE	$-OH$	$-H$	$-H$	>1000. ng (87%)
N,N-DIMETHYLTRYPTAMINE	$-H$	$-H$	$-CH_2-CH_2-N(CH_3)_2$	>1000 ng (90%)
N-ACETYLSEROTONIN	$-OH$	$-H$	$-CH_2-CH_2-NH-CO-CH_3$	>1000 ng
MELATONIN	$-OCH_3$	$-H$	$-CH_2-CH_2-NH-CO-CH_3$	>1000 ng
5-HYDROXYINDOLE ACETIC ACID	$-OH$	$-H$	$-CH_2-COOH$	>1000 ng
5,6-DIHYDROXYTRYPTOPHAN	$-OH$	$-OH$	$-CH_2-CH(NH_2)-COOH$	>1000 ng
TRYPTOPHANE	$-H$	$-H$	$-CH_2-CH(NH_2)-COOH$	>1000 ng
TYRAMINE				>1000 ng
TYROSINE				>1000 ng

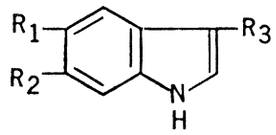

Fig. 3. Inhibition by serotonin analogs of binding of [^{3}H]serotonin by antibody to a complex of serotonin and bovine serum albumin. Conditions of assay are those described in Fig. 2.

Several analogs of serotonin, at various concentrations, were tested to determine the specificity of the radioimmunoassay (Fig. 3). 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 analogs 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, 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 is added.

Although 5-methoxytryptamine is recognized by the antibody, levels of this metabolite in biological fluids can be expected to be low, so that it would not interfere with the radioimmunoassay for serotonin. The enzyme 5-hydroxyindole-*O*-methyltransferase is located exclusively in the pineal gland (*8*). Also, *N*-acetylserotonin is a much better substrate for this enzyme than serotonin itself (*9*). Tryptamine, however, occurs normally in human urine (*10*). Although normal levels are too low to interfere with the serotonin radioimmunoassay, tryptamine levels rise after inhibition of monoamine oxidase (*10*). Under such conditions, the value of the described radioimmunoassay might be limited.

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References and Notes

1. I. H. Page, *Serotonin* (Year Book, Chicago, 1968).
2. G. W. Asheroft, T. B. B. Crawford, D. Eccleston, D. F. Sharman, E. J. MacDousall, J. B. Stanton, J. K. Binns, *Lancet* **1966-II**, 1049 (1966); S. J. Dencker, U. Malm, B. E. Roos, B. Werdinius, *J. Neurochem.* **13**, 1545 (1966).
3. N. S. Ranadive and A. H. Sehon, *Can. J. Biochem.* **45**, 1701 (1967); G. Fillipp and H. Schneider, *Acta Allergol.* **19**, 216 (1964); B. L. Herndon and D. H. Baeder, *Fed. Proc.* **31**, 1764 (1972).
4. J. R. Vane, *Brit. J. Pharmacol.* **14**, 87 (1959); S. Udenfriend, H. Weissbach, C. Clark, *J. Biol. Chem.* **215**, 337 (1955); D. F. Bogdanski, A. Pletscher, B. B. Brodie, S. Udenfriend, *J. Pharm. Exp. Ther.* **117**, 82 (1956); S. H. Snyder, J. Axelrod, M. Zweig, *Biochem. Pharmacol.* **14**, 831 (1965); R. P. Maickel, R. H. Cox, J. Saillant, F. P. Miller, *Int. J. Neuropharmacol.* **7**, 275 (1968).
5. Ott Chemical Co., Muskegon, Michigan.
6. New England Nuclear Co.; specific activity 7.3 c/mmole.
7. J. H. Humphrey and R. Jaques, *J. Physiol.* **124**, 305 (1954); V. Erspamer and R. Faustini, *Naturwissenschaften* **40**, 317 (1953).
8. J. Axelrod and H. Weissbach, *J. Biol. Chem.* **236**, 211 (1961).
9. ———, *Science* **131**, 1312 (1960).
10. A. Sjoerdsma, J. A. Oates, P. Zaltzman, S. Udenfriend, *J. Pharmacol. Exp. Ther.* **126**, 217 (1959).

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