Serotonin and the Cardiovascular System

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Preface

Ever since serotonin was discovered by Dr Irvine Page and his colleagues (see Introduction), its role in health and disease has remained elusive. Indeed it can be characterized as the "parent pauvre" of the catecholamines and other neurohumoral mediators that have key functions in the regulation of the cardiovascular system. Since serotonin is present in the platelets and is released when these aggregate, it has the potential to affect blood vessels in any part of the body, either directly or by augmenting the response to other vasoactive agents. Certainly its actions on the components of the blood vessel wall are complex and the observed responses are the result of many interactions between cells. Interest in the potential role of serotonin was increased by the demonstration that in several cardiovascular diseases, the platelet turnover is exaggerated and that the aging and diseased blood vessel wall becomes hyperresponsive to serotonin. However, it was the advent of new potent serotonergic antagonists and the discovery that one of these, ketanserin, acutely lowers the arterial blood pressure in elderly hypertensive patients that gave credence to the concept of the involvement of serotonin in cardiovascular disease. This discovery provided the momentum for further studies on the subtypes of serotonergic binding sites and the multiple sites of action of serotonin in the cardiovascular system.

In view of the enhanced interest in serotonin, both in health and disease, it is timely to summarize current knowledge and to speculate on its potential role in cardiovascular function. Obviously, it is too early to reach final conclusions, as the points of view on many of the issues covered still diverge, sometimes considerably.

The first chapters of this monograph deal with the biochemistry, physiology, and pharmacology of serotonin and explain its effects on the different target cells that ultimately can alter cardiovascular function. A second series of chapters discusses the possible involvement of serotonin in hypertension and the potential therapeutic use of S_2-serotonergic antagonists as antihypertensive agents. The final part of the book deals with other types of cardiovascular disease in which serotonin could partly contribute to the symptoms.

As always with multiauthored texts, the responsibility for the scientific content rests with the individual authors. Hence, all the statements made are not necessarily endorsed by the editor. His task has been mainly to select the authors, to streamline their texts, to achieve as much as possible uniformity of presentation, and to avoid overt overlap and repetition.

Serotonin and the Cardiovascular System will be of interest not only to physiologists and pharmacologists puzzled by the complexity of the actions of serotonin

and the difficulty of comprehending the consequences of serotonergic blockade, but also to clinical researchers and to the physicians who treat patients with cardiovascular diseases. Indeed, serotonergic inhibitors may well turn out to be a useful way of treating hypertension and vasospastic disorders.

Paul M. Vanhoutte

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Introduction

The Neonatology of Serotonin

We are all part of history, but seldom know it until most of the action has subsided. We speak of "looking back" on history for lessons to guide the future. And so it has been with the isolation and neonatology of serotonin. Thirty-six years ago I participated along with Maurice Rapport and Arda Green in the discovery of serotonin (874). For a few years thereafter I knew everything there was to know about it. Still later I began to know progressively less and less about it. Dr. Green died and Dr. Rapport sought other pastures.

But the subject grew into adolescence at what could only be considered an alarming rate as reflected in an avalanche of papers I was unable to follow. Betty Twarog and I had found serotonin in brain and that simple observation did it! Psychiatrists, neurologists, neurophysiologists soon were in full cry, and we knew we had met our match, and must either give full attention to the field or be merely observers. I confess to regret at having to make the latter decision, since the "chemistry of the brain" was my first major love from my 1928–1931 period in Munich, Germany. History shows that when you are sidelined in research, you are forgotten unless you transform yourself into a disease, like Prinzmetal, Addison, Cushing, or Lou Gehrig—so the name, at least, is remembered.

But how did I become involved with serotonin in the first place? In the early 1930s I was searching for a humoral agent responsible for hypertension. The woods were full of vague suggestions and undefined physiological effects of extracts of plasma and organs. The most immediately disturbing was one that resulted when blood clotted. Plasma carefully prepared had no vasoconstrictor properties, but serum had. This clearly posed a problem for me. Any vasoconstrictor I isolated from blood of hypertensives would always be suspect, because one could never be sure some unseen coagulation had not occurred. Platelets and their secretions are not easily controllable. Before we could progress in the search for a pressor agent in hypertension it was imperative to control this elusive vasoconstrictor resulting from coagulation.

Very briefly, the substance was isolated and its structure identified (Fig. 1). Synthesis by the Upjohn Company followed later. We were made aware only then of the work of Erspamer, a gifted biochemist who had made extracts of stomach and intestine and found that they contracted uterus. Although no purification was achieved, the active substance was labeled "enteramine" and thought to be a "phenolic compound" on the basis of little evidence. I mention this because Erspamer,

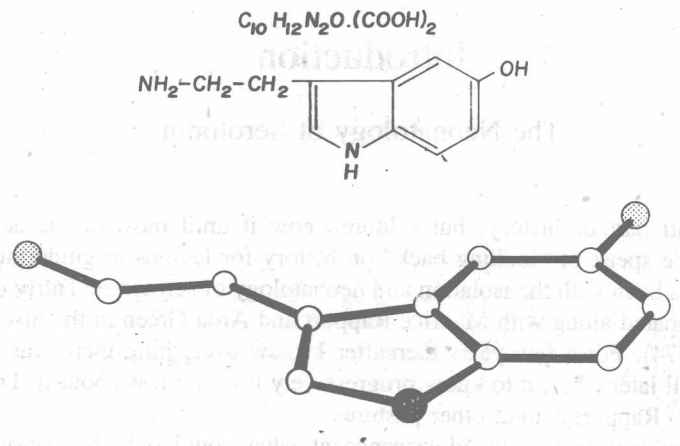

FIG. 1. Chemical structure of 5-hydroxytryptamine (serotonin). The perspective drawing is derived from X-ray crystallographic data (modified from ref. 1046).

unbeknown to us, helped to identify the problem, although he did not solve it. His later work in invertebrate biochemistry proved him to be a man of accomplishment, although he did nothing further with his enteramine except to show in 1952 that it was indeed 5-hydroxytryptamine (319).

Having launched serotonin, it became apparent to me within a year or so that I could not keep up my work in clinical and experimental hypertension and study serotonin as well. After writing reviews covering 153 papers in 1954 (816) and abstracting 530 more papers in 1958 (819), I summarized what I knew in 1968 in a book titled, as you might suspect, *Serotonin* (820).

The serotonin episode has taken about 35 years to develop. What I learned from it was that substances in the body that are nuisances to one person give tenure to others. Clearly, serotonin has been shown to be involved in such varied functions as neural transmission, the symptoms of carcinoid tumors, and endomyocardial fibrosis. Without doubt one of the happiest outcomes for older people like me is the result of experiments in rats showing that on either low or high doses of alcohol, memory becomes poor. Zimelidine, an inhibitor of serotonin reuptake, reversed this alcohol-induced impairment. So it was not Alzheimer's disease after all! If the serotonin neurotransmitter system mediates learning and memory in human beings and simultaneously determines the effects of alcohol, I must say I am impressed.

Serotonin, in short, has taken on a life of its own and we are no longer living together. I have no regrets, because a long life has taught me that the natural history of one's active participation in a discovery is about 5 to 10 years. Then the subject grows complicated, new very bright young faces appear with their better methods and they take over. If they are aware that anything preceded their own work, they give no indication of it—which is nature's way of preventing constipation of the mind.

Serotonin has been slow to grow up and I suspect it is about to enter adolescence and weed-like growth. The important thing now is to keep investigations moving ahead along creative lines. It is easy to fall heir to Brownian movement and perform like the vehicles described in an Arizona newspaper: "The motorized rail car provides service semi-bi-hourly and will run east and west simultaneously."

Irvine H. Page

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Biochemistry of Serotonin

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DISTRIBUTION OF SEROTONIN

Serotonin has a widespread occurrence. In plants, it is present in edible fruits and vegetables but has also been associated with poisonous species (380). In the animal kingdom, serotonin can be detected both in vertebrates and in invertebrates. The important depots for serotonin in mammals are the enterochromaffin cells in gastrointestinal mucosa, the brain (where it is thought to be a neurotransmitter in certain neurons), the pineal gland, where it serves as a precursor of melatonin, and platelets. In addition, serotonergic neurons have been identified in the peripheral nervous system in the gastrointestinal tract in mice, rats, and guinea pigs (392). Significant amounts of serotonin have also been detected in other tissues of mammals, notably in heart, kidney, spleen, and thyroid, although the significance of the presence of serotonin in these tissues is not yet understood. A thorough review of the distribution of serotonin in tissues and body fluids of several mammalian species has been published by Essman (322).

Serotonin is synthesized in most of the tissues in which it is stored and has a rapid turnover in most of these. Half-lives as rapid as 10 to 17 hr have been reported for serotonin in the gastrointestinal tract (320,1061). However, serotonin is not synthesized in platelets, and the serotonin stored in platelets has its origin in other

tissues, notably the gastrointestinal tract (320). The turnover of serotonin in platelets is relatively slow, the reported half-life being between 33 and 48 hr (1061).

Consistent with this rapid turnover of serotonin in most tissues, there are large amounts of 5-hydroxyindoleacetic acid, the major metabolite of serotonin, excreted in urine. Up to 9 mg of 5-hydroxyindoleacetic acid is excreted daily by normal individuals (1058). It is of interest that the amounts of 5-hydroxyindoleacetic acid that are excreted in urine are rather similar to the amounts of vanillylmandelic acid, the major metabolite of norepinephrine (1058). Only small amounts of serotonin (between 10 and 120 μg) are excreted in urine by normal individuals (588).

METHODS OF ASSAY OF SEROTONIN

Overview

Early methods for measuring serotonin were almost exclusively bioassay procedures. These methods were very sensitive but tedious, and they lacked specificity. Presently the major methods for measuring the amine are fluorometric, radioenzymatic, and high performance liquid chromatographic procedures. A radioimmunoassay procedure has been described (847).

Since serotonin is metabolized very rapidly, in many experiments more information can be obtained by measuring 5-hydroxyindoleacetic acid than by measuring serotonin itself. In addition, experimental procedures sometimes involve the administration of 5-hydroxytryptophan (the precursor of serotonin) in an attempt to increase the concentrations of serotonin in tissues. Since under these circumstances it is often desirable to measure all three of the 5-hydroxyindoles, methods for their measurement are briefly mentioned.

Fluorometric Procedures

Fluorometric methods for measuring serotonin are relatively nonspecific and must be preceded by separation of serotonin from other 5-hydroxyindoles and other interfering compounds. Each of the three common methods used for separation (solvent extraction, ion-exchange chromatography, and gel filtration) will be briefly described. This will be followed by a description of major fluorometric methods used for quantitation of serotonin (and of other 5-hydroxyindoles) after separation has been achieved.

Separation

The original method for extracting serotonin from tissues was by solvent extraction as described by Bogdanski, Pletscher, Brodie, and Udenfriend (106), and this method, or some modification thereof, is still in use. Tissues are homogenized in 0.1 N hydrochloric acid and are then centrifuged. Aliquots of supernatant solution are adjusted to pH 10, saturated with sodium chloride, and serotonin is extracted

into *n*-butanol. Aliquots of the *n*-butanol extract are transferred to tubes containing heptane and the serotonin is extracted back into acid. The serotonin in this fraction is assayed by fluorometric techniques. A modification of this extraction procedure is used to measure serotonin concentrations in whole blood (221).

If 5-hydroxytryptophan has been administered in the course of an experiment, it is essential that the *n*-butanol fraction, containing the serotonin, be washed three times with a pH 10 buffer to remove this amino acid. However, these washes can be omitted in determinations of serotonin in tissues from untreated animals since these do not contain sufficient 5-hydroxytryptophan to interfere in the assay.

When ion-exchange chromatography is to be used for separation of serotonin and metabolites, it is necessary that the tissue extract be protein-free prior to separation. Tissues are usually homogenized in 0.4 M perchloric acid and proteins are precipitated by centrifugation. Since 5-hydroxyindoles are not stable in perchloric acid (602), the perchlorates are removed rapidly by adjustment to pH 7 with 1 M potassium hydroxide, followed by centrifugation. Usually cysteine and disodium ethylenediaminetetraacetate are added to the perchloric acid to protect serotonin from oxidation. Alternatively, tissues can be homogenized in a mixture of acetone/1 N formic acid (85:15, vol/vol). The acetone extract is then washed with a mixture of heptane/chloroform (8:1, vol/vol) to remove lipids. The organic layer is removed and the aqueous layer that is formed is evaporated to dryness. The residue is dissolved in water and is ready to be applied to the ion-exchange resin (981).

A weak cation-exchange resin (BioRex 70 or Amberlite CG 50) is used most frequently for the separation of serotonin (981). When the protein-free extract is applied to the resin, serotonin is adsorbed and can subsequently be eluted with acid. 5-Hydroxytryptophan and 5-hydroxyindoleacetic acid are not adsorbed on the resin and are present in the effluent from the column. Catecholamines are adsorbed on the resin and can be eluted together with serotonin, or alternatively, the catecholamines can be first eluted from the resin with 0.67 M boric acid and then serotonin can be eluted with 0.5 N acetic acid (448).

A strong cation-exchange resin (Dowex 50) can also be used for the separation of serotonin and other 5-hydroxyindoles (46). A protein- and perchlorate-free extract of tissues is adjusted to pH 2 and applied to small columns of Dowex 50. All three major 5-hydroxyindoles are adsorbed on the resin at this pH. 5-Hydroxyindoleacetic acid can be eluted first with 60% methanol, and then 5-hydroxytryptophan can be eluted with a neutral buffer. Finally serotonin can be eluted with a mixture of ethanol/1 N hydrochloric acid (1:1, vol/vol).

Separation of 5-hydroxyindoles can also be achieved using gel filtration (296). Protein- and perchlorate-free extracts of tissues are adjusted to pH 2 to 3 and are applied to columns of Sephadex G10. 5-Hydroxytryptophan is found in the effluent from the column. Serotonin is first eluted with a mixture of ethanol/0.01 M HCl (10:80, vol/vol), and then 5-hydroxyindoleacetic acid is eluted with dilute sodium carbonate.

Quantitation

Once the 5-hydroxyindoles have been separated, either by solvent extraction or by column chromatography, they can be quantitated in their respective fractions using fluorescence. All 5-hydroxyindoles fluoresce at neutral pH at A 300 nm F 340 nm. However, the extracts are usually made acid by adding concentrated hydrochloric acid and their fluorescence then measured at A 300 nm F 540 nm. The sensitivity of this assay is reported to be 200 ng for serotonin or for other 5-hydroxyindoles (602).

Two fluorescence procedures exist which increase the sensitivity for the measurement of serotonin. These involve reaction with *O*-phthaldialdehyde (45) or with ninhydrin (1066). The sensitivities of both procedures are reported to be 10 ng for serotonin. The ninhydrin reaction will not proceed in acid solution and it is necessary to adjust extracts to neutrality before proceeding with this reaction. Although all 5-hydroxyindoles react with *O*-phthaldialdehyde the optimum conditions for the reaction vary (1017).

Choice of Methods

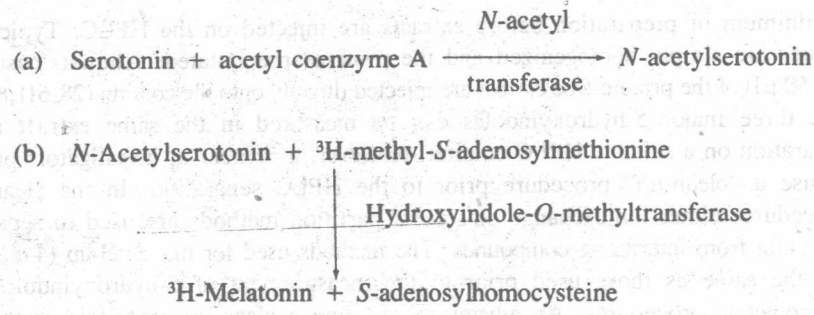
The use of column chromatography is gradually supplanting solvent partition methods for the extraction of serotonin. This is partly because the health hazards associated with handling organic solvents can be avoided. In addition, column chromatography is easier to perform and is generally more economical if only small amounts of serotonin are to be measured. However, as with all analytical procedures, it is essential to monitor recoveries of added authentic standards, and in addition, volumes of washes and of eluents should be checked with each batch of resin that is used.

If column chromatography is to be used, the Dowex 50 separation is a good choice when it is desired to measure all three major 5-hydroxyindoles, since all can be separated using a single column. The Amberlite CG 50 is useful if it is necessary to measure catecholamines as well as serotonin. It is also the resin of choice if low concentrations of serotonin are to be measured, since the monoamine can be eluted from the CG 50 resin with volatile acids, and the eluate can subsequently be lyophilized (448). However, the Amberlite CG 50 is unusually sensitive to high salt concentrations, and if solutions that are applied to this resin have high ionic strength, serotonin will be poorly adsorbed.

In all fluorescence procedures it is necessary to check for "quenching" of the analyte and also to prepare suitable "blank" solutions. For a discussion of the precautions to be taken when using fluorescence, the reader is referred to the classic monograph of Udenfriend (1060).

Radioenzymatic Method

Saavedra, Brownstein, and Axelrod (929) described a method for measurement of serotonin based on the transformation of the amine into melatonin by successive enzymatic reactions as represented by the following equations:



In the first reaction serotonin is *N*-acetylated, and in the second reaction *N*-acetylserotonin is *O*-methylated. *S*-Adenosylmethionine, with a ^{3}H-labeled methyl group, is utilized in the second reaction. The ^{3}H-labeled melatonin that is formed as a result of the two reactions is extracted into an organic solvent and the radioactivity is measured in a liquid scintillation counter.

The *N*-acetyltransferase enzyme used in the first reaction is isolated from rat liver. The hydroxyindole-*O*-methyltransferase used in the second reaction is isolated from bovine pineal glands. It is not necessary to separate serotonin (either by solvent extraction or by column chromatography) prior to performing the assay. The limit of sensitivity of the procedure is 50 pg of serotonin. A modification of the procedure has also been described (505) in which serotonin is chemically *N*-acetylated and only the second reaction is enzymatic. The sensitivity of this modified procedure is reported to be 5 pg of serotonin.

Although this procedure is very sensitive, it has not been widely used. It shares the disadvantages of many enzymatic procedures that the enzymes are intrinsically unstable and are not readily available. In addition, the high cost of the radiolabeled *S*-adenosylmethionine makes the assay expensive.

High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) can be used for the measurement of 5-hydroxyindoles since these are easily oxidizable and are electroactive. In practice the 5-hydroxyindoles are separated on an HPLC column. When eluted from the column the 5-hydroxyindoles are oxidized at an electrode surface under controlled potential conditions. The current resulting from the transfer of electrons across the interface of the electrode and the solution is directly proportional to the concentration of 5-hydroxyindole eluted. The retention time of the analyte on the column is characteristic of the compound and gives some specificity to the method. In addition, the electrochemical detector is selective in that only compounds that are electroactive at a given potential are oxidized. For a discussion of the principles of electrochemical detection the reader is referred to the review by Kissinger, Bruntlett, and Shoup (570).

Several methods have been described for the measurement of 5-hydroxyindoles by HPLC with electrochemical detection (28,720,782,1124). Tissue samples require