SURVEY OF CHEMICAL AND PHYSICAL METHODS FOR MEASURING CATECHOLAMINES

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We must admit that bioassay procedures for physiologically active substances can be sensitive and specific and from a biologist's standpoint even fairly simple. In the field of catecholamines much of our present knowledge was attained by bioassay procedures. However, what may be simple to a physiologist is either inconvenient or presents difficulties to a chemist. With the influx of biochemists into the field of catecholamines it is not surprising that physical and chemical procedures for estimating catecholamines in tissues have become more and more popular.

The first attempts to measure catecholamines in blood by chemical methods occurred almost 25 years ago. Since then many procedures utilizing a variety of principles have been reported. The technique of isolating them by using an adsorbent such as alumina was devised by Shaw in 1937 (14). With some variations this is the most widely used procedure today. Following isolation many procedures have been utilized. The most popular colorimetric method was based on the blue color obtained with arsenomolybdate (18). Even methylene blue reduction by liver homogenates catalyzed by adrenaline was used to measure adrenaline in blood (8).

The values reported by these methods were usually ridiculously high so that in 1941 Bloor and Bullen (1) published a critical evaluation of the most sensitive of these methods and concluded that "if present at all the adrenaline content of venous blood of man and dog is less than 0.001 μg/ml." Amazing as it may sound, 10 years after this in 1951 (5), just a few years ago, papers were still appearing in biochemical journals utilizing colorimetric procedures and reporting peripheral levels of adrenaline as high as 10 μg/ml.

The need for more sensitive and specific methods for catecholamines brought fluorescent procedures into use quite early. As early as 1940, Hueber (7) reported making use of the evanescent yellow-green fluorescence which appeared when adrenaline solutions were made alkaline, for assay in blood. Again many investigators made use of this procedure for a variety of physiological, pharmacological and clinical studies. In this country Heller *et al.* (6) studied the procedure and concluded that some oxidation product related to adrenochrome was responsible for the observed fluorescence but reported that the method as used was not sufficiently sensitive or specific to measure levels of circulating catecholamines. As used by Heller and his predecessors, the method required the measurement of a fluorescence which persisted for seconds and the duration of which was sensitive to pH, dissolved oxygen and the presence of reducing agents. It is not surprising that this procedure did not meet with wide acceptance.

In subsequent studies by Lund (9–12) the mechanism of this oxidation and re-

arrangement of adrenaline in alkaline solution was elucidated thus providing the information necessary to develop the trihydroxyindole procedure. Modifications of this procedure and its application to physiological and clinical problems are discussed by von Euler (4).

It is now obvious that of all the techniques available, only fluorescence offers the required sensitivity. With the development of commercial spectrophotofluorometers it is now possible to attain specificity as well. Because of the dependence of catecholamine assay on fluorimetric methods we have thought it suitable for Dr. Robert Bowman, who was responsible for the design and development of the spectrophotofluorometer (2), to discuss problems relating to both instrumentation and the phenomenon of fluorescence itself.

Since we are to be dependent on fluorimetric methods, it would be desirable to discuss relationships between molecular structure and fluorescence in solution. First, in order to emit fluorescence light a molecule must absorb light. However, not all substances which absorb light emit fluorescence detectable with the available instruments. Thus benzene does not exhibit detectable fluorescence. However, phenol does and so does aniline; phenolic ethers and alkylated amines are also fluorophores. Polyphenols also fluoresce and since the catechols are in this class one may ask why chemical manipulations are needed to measure catecholamines fluorimetrically.

Catecholamines, in their native form, do fluoresce. Epinephrine, norepinephrine and dopamine are all activated at 285 mμ and fluoresce at 325 mμ, fluorescence being maximal at pH 1 (3). These fluorescence characteristics were discovered following the development of the spectrophotofluorometer. Thus far no one has made use of this native fluorescence for assay of catecholamines in tissues. One problem that arises is that if all the catechols have the same fluorescence characteristics then no distinction can be made from one to another. However, this in itself may prove useful since it should then be possible to measure total catecholamines in tissues.

Since the catecholamines are already fluorophores what is the need for further chemistry? Originally this was necessary in order to utilize the available instruments. Since these could activate only at certain wavelengths near the visible region and could detect only emitted fluorescence in the visible region it was necessary to convert the molecule to some form which would meet these requirements. Many of the rules which apply to light absorption also apply to fluorescence. The introduction of additional conjugated double bonds into the molecule by shifting the absorption towards the visible region also shifts the activation towards the visible. Thus by a series of oxidations and rearrangements catecholamines are converted to polyhydroxyindoles which are the fluorophores in the method of Lund (10). These compounds absorb light near the visible range and emit visible fluorescence.

In general, polyaromatic and polycyclic phenols or amines will fluoresce in the visible region. Hydroxyquinolines, naphthols and naphthylamines produce visible fluorescence as do hydroxyindoles.

The second of the two procedures for catecholamines involves condensation

with reagents leading to visibly fluorescent polycyclic compounds. The observation by Natelson (13) that ethylenediamine condenses in such a manner with catecholamines was subsequently utilized by Weil-Malherbe (16) to develop the quinoxaline procedure which he will discuss in this symposium (17). Condensation with diamines other than ethylenediamine has been reported (15) but has not been used to any great extent.

The latter two procedures have been so widely utilized, both in their original forms and in so many modifications, that it has become difficult for even an expert to determine which modifications are most suitable for a given problem. In the case of the trihydroxyindole method arguments have arisen as to the best oxidant, the best pH values at which to carry out oxidations and the optimum means of measuring the resulting fluorescences. Similarly differences of opinion have arisen as to the most suitable modifications of the quinoxaline method: should the reaction be carried out in the dark, and what are the best means for distinguishing the epinephrine product from the norepinephrine product?

Aside from these considerations, novices in the field of fluorescence may not realize certain important considerations which, if not taken into account, may lead to peculiar and unexplainable results no matter what procedure is used. One of the most important factors to be considered is that all solutions, including the blanks, emit some light when activated. This light is composed of some true fluorescence and some scattered light. The limits of sensitivity of a given procedure are governed by this blank fluorescence. Whether one sets this blank to zero or reads it and then subtracts it, it is nevertheless there. However, unless the magnitude of this blank is known it is impossible to evaluate the precision of a given procedure. Thus, if data are presented showing galvanometer deflections over the reagent blanks of sample a, 20, and sample b, 40, these values may be satisfactory. However, the actual data may be: blank, 120; sample a, 140; and sample b, 160. With this information the data will not look so good. If, furthermore, the variation of the blank is 120 ± 20 then the data may be meaningless. I am afraid that in stretching to the limits of sensitivity, some of the data obtained with the fluorescence procedures may fall within this latter category. I hope that this problem will be considered in discussing applications of these methods.

Another point to be considered is the presence of other substances in the final extract or reaction mixture which, while not fluorescing themselves, do absorb light. When appreciable amounts of such materials are present they act as filters and remove either activating light or fluorescence light. With visible fluorophores it is merely necessary to see that the solution is colorless to make sure that fluorescence is not being filtered out. However, the presence of substances which absorb the activating light below 400 mμ can only be detected by use of a spectrophotometer. Excessive material of this type, such as is frequently found in urine extracts, causes appreciable loss in activating light and therefore in emitted fluorescence. If not present in excessive amounts internal standards, prepared by adding authentic material to the extracts, can correct for such light loss. Excessive turbidity scatters the activating light and therefore lowers the intensity. This also

decreases the amount of fluorescence. With the spectrophotofluorometer, light scattering is evident and can be measured. One should take cognizance of it. A more thorough discussion of such considerations concerning fluorescence in solution was presented by Duggan *et al.* (3) in 1957.

It is our hope that this symposium will make it possible to arrive at general agreements as to the most desirable modifications of the chemical procedures and to determine practical limits of sensitivity, specificity, applicability and reproducibility.

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