THE DEVELOPMENT AND APPLICATIONS OF THE TRIHYDROXYINDOLE METHOD FOR CATECHOLAMINES

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The appearance of a yellow-green fluorescence in adrenaline solutions after addition of strong alkali was first reported by Loew in 1918 (24). This reaction was further studied by Paget (32) who observed that it was apparently specific for adrenaline. Barker *et al.* (1) noticed the high sensitivity of the reaction which was further studied by Gaddum and Schild (18), who found that the reaction required oxygen and that noradrenaline gave a much weaker reaction than adrenaline. These authors also considered the possibility of using the reaction for assay purposes. Attempts to utilize this sensitive method for the quantitative estimation of catecholamines were, however, largely unsuccessful owing to the instability of the fluorescent compound (36). The formation of the fluorescent compound occurs more rapidly than the degradation, which causes the fluorescence to increase at first and then to decrease after having reached a maximum. The application of the reaction at this stage, particularly for the estimation of adrenaline in blood, has given widely varying results and the different procedures used during this period will not be considered further in this review.

Nature of fluorescent compound

The formation and the nature of the fluorescent compound was studied by several authors in the early 1940's (22, 44, 48). It was then believed to be either the leucoadrenochrome in ionic state or some compound formed along with adrenochrome. In 1948 the studies of Ehrlén (10) led him to conclude that the formation of the fluorescent compound was due not to a reduction but to a rearrangement of the adrenochrome molecule. Wiesner (49) had estimated the redox-potential for the equilibrium: leucoadrenochrome $\rightleftharpoons$ adrenochrome + 2 H^+ + 2 e to be +0.044 V at pH 7. From this it was inferred that leucoadrenochrome is oxidized to adrenochrome in alkaline solution in the presence of oxygen. Adrenochrome is subsequently transformed to the fluorescent compound. In conformity with this theory a solution of leucoadrenochrome develops a red color of short duration concurrently with the development of fluorescence on addition of alkali. Ehrlén (10) gave the correct formula for the fluorescent compound of adrenaline as 1-methyl-3,5,6-trihydroxyindole. Adrenochrome itself gives no fluorescence. A similar rearrangement had previously been shown to occur in the melanization process of tyrosine (Raper, 38). When an oxidant such as potassium ferricyanide is added to a solution of the fluorescent compound the fluorescence disappears, probably by oxidation to the corresponding quinone.

On the basis of these considerations Ehrlén (10) developed a method for the quantitative estimation of adrenaline. The important new point was the addition

of a reducing agent by which the further oxidation of the fluorescent compound was prevented. At about the same time Harley-Mason (20) described the formation of a strongly fluorescent compound of adrenaline to which he ascribed the formula 2,3,5,6-tetrahydroxy-N-methyl-2,3-dihydroindole. The zwitterion which occurs on reduction of adrenochrome has not been isolated but gives rise to the fluorescent compound on addition of alkali. By acetylation with acetic anhydride-pyridine a dehydration product was obtained, 3,5,6-triacetoxy-N-methylindole. The correct formula for the fluorescent compound was later recognized by Harley-Mason (21). Fischer (16) reported in 1949 that the fluorescent compound obtained by addition of alkali to a solution of adrenochrome could be extracted with ether if the alkali was neutralized immediately after its addition. High concentrations of the fluorescent compound could even be crystallized directly on cautious neutralization of the solution with acid. He reached the conclusion that the fluorescent compound had the formula proposed by Harley-Mason in 1948 (20). An interesting finding was soon after reported by the Belgian group as Fischer *et al.* (17) showed that adrenochrome may be isomerized to 3,5,6-trihydroxy-1-methylindole by treatment with zinc acetate or aluminium chloride in neutral solution. In this way the results of Utevskî (44) may also be explained since he found fluorescence when adrenaline was adsorbed on an alumina column.

At about the same time Lund (26) showed that addition of strong alkali to an oxygen-free adrenochrome solution causes a change of color to yellow, accompanied by development of strong fluorescence. Addition of acid at this point alters the fluorescence from yellow-green to green. At the neutral point a yellow crystalline precipitate is formed. In a later paper (27) the name adrenolutine was proposed for the fluorescent compound formed from adrenochrome. It was suggested that the fluorescent compound had the formula 1-methyl-5,6-dihydroxyindoxyl plus 1 mol of water as in the formula of Harley-Mason (20). Lund (27) also prepared the triacetyladrenolutine according to Harley-Mason (20). From the studies of Ehrlén, Harley-Mason, Fischer and Lund in 1948–49 it thus became clear that the fluorescent compound formed by addition of alkali to adrenochrome is an isomeric transformation product having the composition of 1-methyl-3,5,6-trihydroxyindole. The rearrangement is sometimes referred to as autoreduction.

Further studies of the formation of lutines have been reported by Bu'Lock and Harley-Mason (6) including those formed from noradrenaline, α-methylnoradrenaline and N-isopropylnoradrenaline. It was possible to prepare a crystalline semicarbazone from N-isopropylnoradrenaline by oxidation with potassium ferricyanide buffered with sodium hydrogen carbonate. When treated with alkali this compound gave a deep yellow solution with a strong green fluorescence. Cautious acidification with acetic acid yielded a precipitate from which 3,5,6-trihydroxy-1-isopropylindole could be isolated. The less stable quinones obtained from noradrenaline and α-methylnoradrenaline gave no fluorescence on addition of alkali, however. By oxidation of these amines with potassium iodate the 2-iodochromes were formed. Since treatment of adrenochrome with acetic

anhydride and pyridine caused rearrangement as well as acetylation, this reaction was studied on the iodoquinones. From the resulting iodoindoles the iodine atom was removed with zinc dust and acetic acid. Although alkaline hydrolysis did not yield sufficient amounts of the dihydroxyindoxyls for isolation there was little doubt that such products were formed since the yellow solutions showed the typical green fluorescence.

Zinc acetate treatment as described by Fischer *et al.* (17) for adrenochrome has also been applied to the iodoquinones by Bu'Lock and Harley-Mason (6) with similar results. When the procedure is followed by treatment with sodium dithionite the iodine atom is removed. The indoxyl could not be isolated, however, from the fluorescent solution obtained in this way from 2-iodonoradrenochrome.

Application of trihydroxyindole (THI)-method for the estimation of catecholamines

Although the THI-method had been applied in its primitive form by several authors for the quantitative estimation of adrenaline, it was the all-important method for stabilization of the indoxyls introduced by Ehrlén (10) which made the method truly useful for assay purposes. Ehrlén was also the first to use the technique so developed for the estimation of adrenaline in solutions containing procaine. In his experiments potassium ferricyanide was used as oxidant.

Using a procedure based on the same principle, Lund (28) developed the method and adapted it for the estimation of adrenaline and noradrenaline in blood. Manganese dioxide was used as oxidizing agent.

Blood plasma. It has been reported by several investigators that blood plasma or even dialysates of blood plasma depress the fluorescence obtained from adrenaline added to blood (31). It therefore became of importance to purify the amines before the formation of lutines to be determined fluorimetrically. This is achieved with the alumina adsorption method originally described by Shaw (41). Following the technique used by Lund (28), the catecholamines are as a rule adsorbed on aluminium oxide and subsequently eluted with acetic acid.

In order to estimate adrenaline and noradrenaline in a mixture, Lund (28) utilized the previous finding (14) that the oxidation of noradrenaline proceeds very slowly at low pH values at which adrenaline still is rapidly oxidized. By oxidation with manganese dioxide at pH 3.0 and at pH 6.5 adrenaline on the one hand and noradrenaline and adrenaline on the other are oxidized and their lutines subsequently formed by adding alkali. The sensitivity of the method did not allow estimation of the concentrations of adrenaline and noradrenaline in normal blood although estimations could be made during special conditions (28).

The technique for the estimation of catecholamines in plasma has since been improved in certain respects and the sensitivity and precision considerably increased. Instead of manganese dioxide other oxidants such as iodine or potassium ferricyanide may be used. In this connection the modifications introduced by Price and Price (37) and by Cohen and Goldenberg (7, 8) should be mentioned particularly. Some of the major points in their techniques will be briefly mentioned.

Price and Price differentiate between the lutines of adrenaline and noradrenaline by using different filter sets rather than by oxidation at different pH. Furthermore, the fluorescence is increased by adjusting the pH of the reaction products from the strongly alkaline reaction to pH 5. Finally the blank is prepared by adding ascorbic acid, followed by ferricyanide and sodium hydroxide. The plasma catechols, adsorbed on an alumina column, are eluted with acetic acid, oxidized with potassium ferricyanide at pH 6 and transformed to the lutines by strong alkali in presence of ascorbic acid. After adjustment to pH 5 the fluorescence is read at a fixed interval after addition of alkali, using the filter combinations 400 mμ Farrand interference and 436 mμ Farrand interference, both plus Wratten no. 35 (exciting), and 500 mμ Farrand interference plus Wratten no. 57 (emitting). The amounts of adrenaline and noradrenaline are calculated according to the same principle as given below. The recovery obtained with this technique is around 80% and the sensitivity high, allowing estimations with 30 ml blood. The amounts found in normal arterial plasma were 0.10 $\pm$ 0.10 μg/l of adrenaline and 0.20 $\pm$ 0.12 μg/l of noradrenaline. It is of interest that the concentration of adrenaline in antecubital venous plasma was lower and that of noradrenaline higher than in arterial plasma.

The multiple-filter technique employed by Price and Price (37) has also been utilized in a study published almost at the same time by Cohen and Goldenberg (7, 8). Oxidation of the amines in the eluate from alumina was achieved with manganese dioxide and the lutines were estimated by the fluorescence read with two filter sets (405 mμ excitation and Ilford-Bright 623 secondary filter; 436 mμ excitation and Corning 3486 secondary filter, respectively). With these filter sets the relative fluorescence of the two lutines differed sufficiently to allow estimation in a mixture according to the equations:

$$A = y\,Na + x\,Ea$$

$$B = Y\,Nb + x\,Eb$$

x and y: amounts of adrenaline and noradrenaline,

A and B: fluorimetric readings for filter sets a and b.

Ea, Eb, Na and Nb: fluorescence per μg with filter sets a and b for adrenaline and noradrenaline respectively.

The recoveries of adrenaline and noradrenaline added to plasma were 70 to 90%, suggesting that the amounts obtained in plasma would not be much higher than those observed. The amounts found with this technique in 65 samples of normal human venous plasma (uncorrected) were 0.30 $\pm$ 0.07 μg noradrenaline and 0.06 $\pm$ 0.05 μg adrenaline per litre plasma. These figures are not in disaccord with those found by Price and Price (37) but are considerably lower than those found by Weil-Malherbe and Bone (46).

Weil-Malherbe and Bone (46) have compared the results obtained with the ethylenediamine condensation method and the trihydroxyindole method. They found at every stage of purification a close agreement between the results obtained with the two methods. Moreover it is stated that 3-hydroxytyramine

(dopamine) and acid catechol metabolites are absent in plasma and therefore cannot interfere with the ethylenediamine method. Column chromatography of beef plasma eluates from alumina has, however, suggested the presence of dihydroxyphenylacetic acid (dopac) in plasma but not dopamine (13). Spectrophotofluorometer curves obtained from the corresponding fractions are also consistent with the view that dopac is present in plasma. However, it should be mentioned that in the studies of Weil-Malherbe and Bone (46) acetic acid was used for elution from the alumina, while sulfuric acid was used in the experiments made in our laboratory. Certain observations indicate that the elution of dopac and dihydroxymandelic acid (doma) may be less complete with acetic acid than with sulfuric acid.

The trihydroxyindole method has also been applied for catechol estimation in plasma, using the Aminco-Bowman spectrophotofluorometer, by Bertler *et al.* (3) with good results. These authors, like Weil-Malherbe and Bone (46), use zinc sulfate routinely as oxidation catalyst also at pH 6.

Urine. The trihydroxyindole method has been applied to urine by several investigators because it is a rapid and simple method for estimation of catecholamine excretion. Oxidants used have been MnO_2 (9, 19, 29, 30, 33, 34, 35), iodine (43) or potassium ferricyanide (2, 11, 12, 15, 39, 40, 45, 47). After adsorption of the urine catechols on alumina, either by mixing or by passage through a column, and elution with sulfuric, oxalic or acetic acid, the catechols in the eluate are oxidized and transformed to the fluorescent lutines. In order to differentiate between adrenaline and noradrenaline, oxidation may be carried out at pH 3 to 3.5 and 6 to 6.5 respectively. The multiple filter technique has been adapted in a modification of an earlier method used in our laboratory (15). When oxidation is performed at a different pH it is essential that the eluate be adjusted to 6 to 6.5 under adequate control since addition of buffer alone may give a slightly lower pH at which the oxidation proceeds more slowly. It has been observed that adjustment of pH with sodium phosphate, sodium bicarbonate or ammonia causes smaller losses due to local alkalization in the solution than 0.5 to 1 N sodium hydroxide.

At pH 3 to 3.5 the oxidation of adrenaline with potassium ferricyanide takes place very slowly. Addition of zinc sulfate acting as a catalyst makes the oxidation process proceed faster. In the presence of zinc sulfate the oxidation is completed in 2 minutes at pH 6, and in 3 minutes (for adrenaline) at pH 3.5. The amount of potassium ferricyanide for optimal oxidation should as a rule not exceed 500 μg while the amount of zinc sulfate seems to be less critical. It is, however, important that the amount of ascorbic acid used in conjunction with the alkali be not too great since this causes a reduction of the fluorescence. The optimal results were obtained with 1 to 2 mg ascorbic acid. The stability of the fluorescence developed is satisfactory during a period of about 1 hour at room temperature. The influence of illumination in connection with the readings in the fluorometer must be considered. The necessity of using demineralized water and fresh reagents should be emphasized. In all catechol estimations in urine the addition of ethylenediamine tetraacetic acid has been found indispensable. When

using eluates with acetic acid the buffering capacity of the eluate may necessitate the use of larger amounts of the ascorbic acid-alkali than the standard volume (15). Some further methodological points are also mentioned in this paper.

For the quantitative estimation of catechol compounds in urine, chromatographic separation on a column using n-butanol-hydrochloric acid–acetic acid as solvent, and subsequent application of the THI-method and assay either with multi-filter technique or on the Aminco-Bowman spectrophotofluorometer, has proven useful. In discussing the applicability of the THI-method for the estimation of catecholamines in urine it is also necessary to consider the possible interference of other catèchols. Urine is known to contain fairly large amounts of dopamine. This amine gives only a weak fluorescence when ferricyanide is used for oxidation, while on oxidation with iodine it gives even stronger fluorescence than adrenaline and noradrenaline (42). Dihydroxyphenyl-acetic acid gives only negligible fluorescence in comparison with adrenaline and noradrenaline.

Tissues. The THI-method has been used in several instances also for tissue extracts. After precipitation of proteins with acid alcohol, trichloroacetic acid or perchloric acid (4, 5) the extracts are either adsorbed on alumina or purified by means of ion exchange resins. Carlsson's group (4, 5) have used a column of Dowex 50 × 8 treated with 2 N hydrochloric acid and sodium acetate buffer pH 6.0. By using 1 N hydrochloric acid followed by 2 N acid for the elution, adrenaline and noradrenaline could be separated from dopa and dopamine. The former compounds are estimated by the THI-method after oxidation with potassium ferricyanide in the Aminco-Bowman spectrophotofluorometer.

Shore and Olin (42) extracted the catecholamines with butanol from a saturated solution of sodium chloride and utilized iodine as oxidant. The catecholamines from various tissues were identified and assayed by means of the Aminco-Bowman spectrophotofluorometer.

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