THE FLUORIMETRIC ESTIMATION OF CATECHOL COMPOUNDS BY THE ETHYLENEDIAMINE CONDENSATION METHOD

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The formation of a fluorescent derivative from adrenaline and ethylenediamine was first described by Natelson, Lugovoy and Pincus (26). These authors also found that ethylenediamine could be replaced by other primary amines, either aliphatic or aromatic; they stressed the gain in specificity when radiation of 435 mμ was used for activation rather than shorter wavelengths and they showed that the fluorescent reaction product was extracted by amyl alcohol. Several details of their procedure were subsequently modified by Weil-Malherbe and Bone (34).

Natelson *et al.* were not the first to use this type of reaction for the detection of catechols. Two years earlier Wallerstein, Alba and Hale (33) found that *o*-phenylenediamine and oxidized catechols form fluorescent condensation products, extractable with n-butanol or a mixture of n-butanol and ethyl acetate. Chodat (4), in 1912, studied color reactions between *o*-benzoquinone and amino acids.

An important clue to the mechanism of these reactions is given by the work of Kehrmann and Cordone (19) who, in 1913, showed that 4,5-dianilino-1,2-benzoquinone was formed from catechol and aniline (Fig. 1, a). Pugh and Raper (28) and Hackman and Todd (14) confirmed and extended these results (Fig. 1, b, c, d); it appears from their studies that the preferred reaction is an oxidative condensation in the two positions *para* to the quinonoid oxygens. Only when substituents are present in these positions can a nonoxidative condensation take place between the amino group and the quinonoid oxygen, analogous to the formation of a Schiff's base (Fig. 1, b, c, d). Similar reactions occur between amino acids and *o*-benzoquinone (Fig. 1, e, f).

In the reaction of ethylenediamine with catechol, both types of condensation are found (Fig. 2, a) (16). Adrenaline, on oxidation, very rapidly cyclizes to adrenochrome in which neither of the positions *para* to the quinonoid oxygens is available for substitution. Consequently condensation takes place with the quinonoid oxygen groups. The general ring configuration of the product is identical with that suggested by Weil-Malherbe and Bone (34) but further oxidation leads to a fully aromatic compound (Fig. 2, b).

The main product formed from noradrenaline is identical with that obtained from catechol (15). This surprising result may, according to Harley-Mason, be explained as follows: noradrenaline quinone cyclizes much more slowly than adrenaline quinone, with the result that ethylenediamine substitutes in position 6. The next step is a cyclization of the ethylenediamine side chain with the elimination of the β-ethanolamine side chain (Fig. 2, c). The expulsion of the original side chain is possible only if there is a hydroxyl group at the β-carbon; the reaction may therefore be regarded as the reversal of an aldol condensation. In the reaction of ethylenediamine with dopamine the side chain is not eliminated.

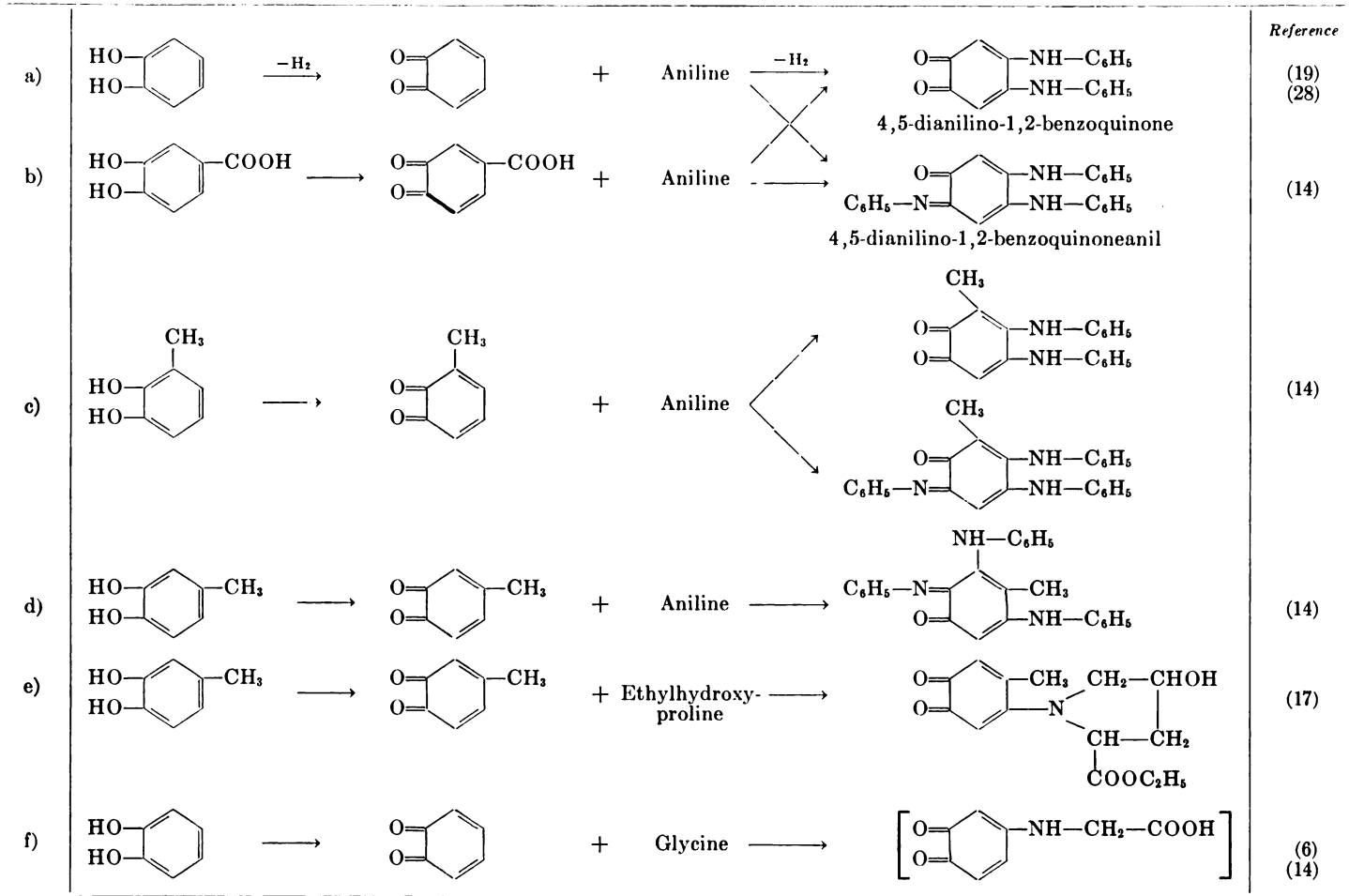

FIG. 1. Reactions of amines with catechols.

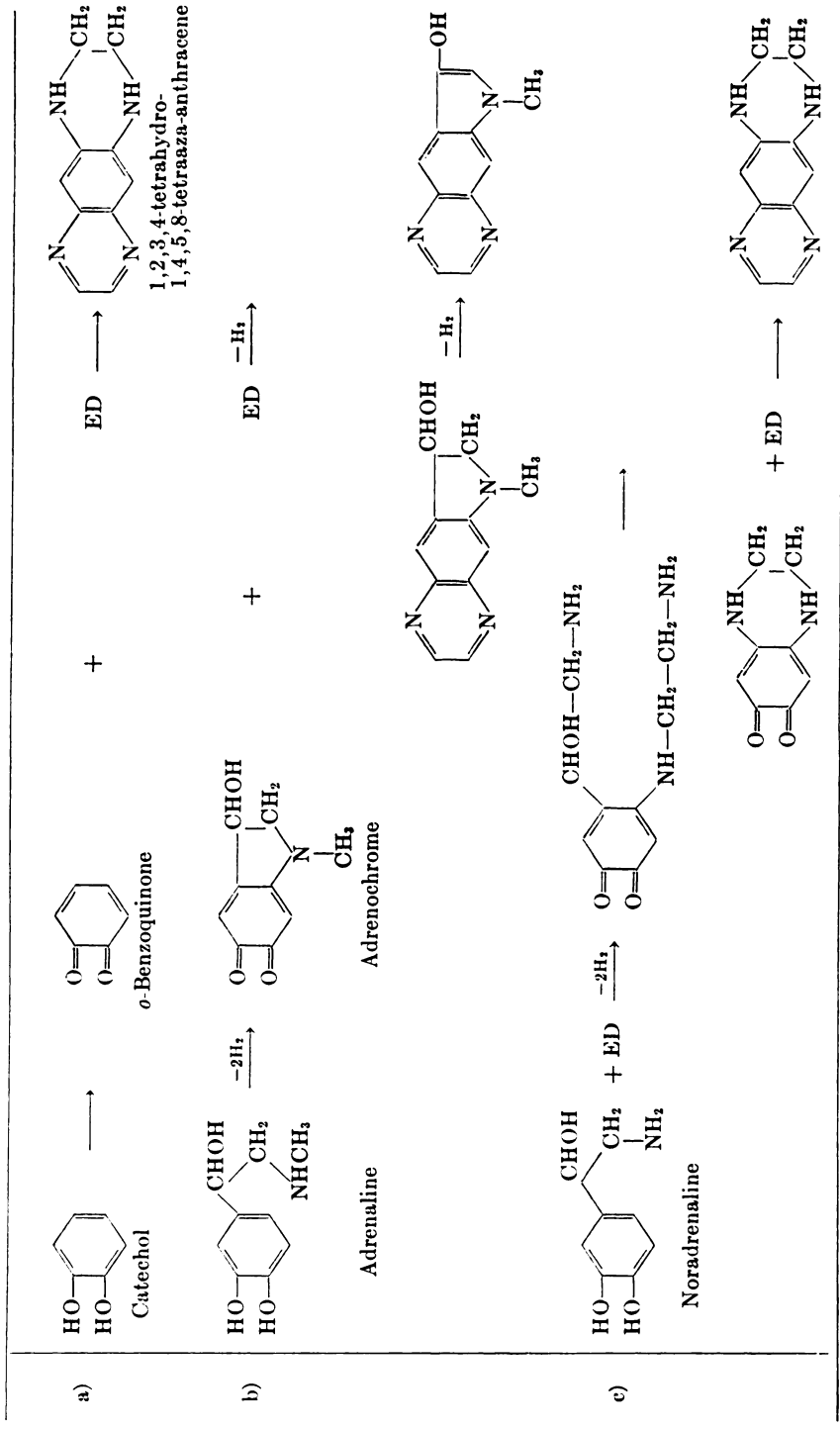

FIG. 2. Reaction of ethylenediamine (ED) with catechols (15, 16).

Although these reactions probably describe the principal events of the condensation, various side reactions may occur. Investigation of the reaction product formed from adrenaline or noradrenaline by paper chromatography or paper electrophoresis has revealed the existence of several components. Burn and Field (3) found two fluorescent derivatives of adrenaline and two of noradrenaline, while Young and Fischer (43) reported the formation of three derivatives from adrenaline and of two from noradrenaline. Nadeau and Joly (24), lastly, detected not less than 10 different substances derived from adrenaline and about the same number from noradrenaline. According to Young and Fischer (43) a compound with an absorption peak at 415 mμ is predominant when the condensation of adrenaline is performed under the conditions recommended by Weil-Malherbe and Bone (34), but when the concentration of ethylenediamine is decreased, compounds absorbing at lower wavelengths are formed in increased amounts. Naturally, workers who aim at isolating the reaction product tend to increase the ratio of adrenaline to ethylenediamine. Thus, in the experiments of Burn and Field (3), the concentration of noradrenaline was about 1000 times higher and that of ethylenediamine 2 to 20 times lower than the concentrations specified by Weil-Malherbe and Bone. If the reaction is carried out under the proper conditions the fluorescence spectra of adrenaline and noradrenaline appear to be symmetrical curves with single peaks, which would not be the case if we were dealing with a multitude of compounds.

Methodology

Various modifications have been proposed concerning the temperature, pH and length of incubation to be used for the condensation. The conditions originally proposed were found optimal for adrenaline (2). Later, the reactions of noradrenaline and 3-hydroxytyramine (dopamine) with ethylenediamine were studied, at 50° and 100°C (37: Fig. 3). Raising the temperature to 100°C did not increase the fluorescence intensities significantly; at 50°C, an approximately stable level of fluorescence was reached between 20 and 40 minutes. It thus appeared that the conditions adopted for the estimation of adrenaline were also suitable for that of noradrenaline and dopamine. Contrary to our findings, Burn and Field (3) as well as Erne and Canbäck (8) consider that solutions containing noradrenaline must be heated for 2 hours at 50°C before fluorescence is fully developed. Kägi *et al.* (18) found that, although noradrenaline solutions yield maximum fluorescence after 20 minutes at 50°C, a temperature of 63°C is required if noradrenaline is added to, or present in, a plasma eluate.

According to Erne and Canbäck (8) ammonium molybdate has a catalytic effect on the development of fluorescence from noradrenaline. When the molybdate effect was examined in our laboratory, it was found to be insignificant at 50°C, whether adrenaline, noradrenaline or dopamine was present; at 100°C the fluorescence formed from noradrenaline was increased by molybdate, but it was very unstable and rapidly faded (37).

We have studied the effect of numerous ions on the development of fluorescence from adrenaline and, although many were found to depress the fluorescence

intensity, none was found to increase it (42). It has been claimed (20, 25, 32) that adrenaline yields more fluorescence in a solution of acetic acid which has been passed through a column of alumina than in a similar solution not thus treated, and Nadeau *et al.* (25) have attributed this effect to dissolved aluminum ions. We have been unable to confirm either the effect of the alumina column or that of aluminum ions (40).

Another point which has been stressed by several authors is the light sensitivity of the noradrenaline derivative. Goldfien and Karler (12) have presented fluorescence spectra obtained 1) by carrying out the reaction in darkness, 2) after exposure to daylight for 5 minutes and 3) without exclusion of daylight at any time; the curves show a progressive decline of fluorescence intensity. We have made similar observations and made it a rule to leave the tubes exposed to diffuse daylight for at least 30 minutes before fluorimetry, a procedure also recommended by Gray and Young (13). In our experience readings remain stable for at least 24 hours under these conditions and the fluorescence is not unduly sensitive to irradiation during fluorimetry. This procedure seems to us preferable to that advocated by Aronow and Howard (1) who work in a red-illuminated dark room. Although a higher fluorescence is thereby obtained from noradrenaline, it is also more readily affected by the blue light of the exciting beam.

The light sensitivity of the noradrenaline derivative and perhaps other condensation products puts a limit to the intensity of the primary light. It is, in my opinion, a bad feature of most commercial fluorometers to have the primary beam sharply focused on a point in the fluorescent solution. It is not only uneconomical since it leaves a large part of the fluorescent solution outside the exciting beam, but the irradiation of the exposed regions may also be unnecessarily intense. It seems to me preferable to use a beam of parallel light for excitation, wide enough to fill the bulk of the solution, though suitably screened so as not to strike the walls of the cuvette. Furthermore, light scatter and reflections are greatly reduced, if the exciting beam enters through the bottom of the cuvette, owing to the absence of an opposing glass face.

A further controversial point is the need for oxygenating the solution during the heating stage. This has been recommended by Burn and Field (3) and by Townsend and Sourkes (31), but Young and Fischer (43), in agreement with our own experience, found no significant effect.

Finally, the possibility of replacing ethylenediamine by other amines, already demonstrated by Natelson *et al.* (26), will be mentioned. At an early stage of our work we tested several amines, particularly *o*-phenylenediamine and 2,3-diaminonaphthalene, but were discouraged by our inability to reduce the high fluorescence of the blanks. Sulkowitch (30) has since described a procedure in which 2,3-diaminonaphthalene is condensed with oxidation products of catecholamines in acid solution (pH 2.5). High sensitivity is claimed for this method.

Differentiation between adrenaline and noradrenaline

If the condensation products of adrenaline and noradrenaline are the only fluorescent substances present, they can be estimated differentially by optical

TABLE 1

*Fluorescence of catechol derivatives in the ethylenediamine reaction**

Adrenaline	100	Catechol	96
Noradrenaline	35	Adrenalone	0.6†
Dopamine	36	3,4-dihydroxyphenylalanine	0.6†
Isoprenaline	56	3,4-dihydroxyphenylacetic acid	3.8†
Epinine	48	3,4-dihydroxymandelic acid	4.0

* Relative fluorescence, measured with yellow filter (Chance OY 4).

† Aqueous phase more strongly fluorescent than isobutanol phase.

means. The peak fluorescence of the adrenaline derivative is found at about 530 mμ, that of the noradrenaline derivative at about 485 mμ. At wavelengths in the region of 600 mμ the fluorescence intensity of the adrenaline derivative is about four times that of the noradrenaline derivative; at 510 mμ the ratio is 0.71 (20). By measuring the fluorescence at approximately these two wavelengths the concentration of adrenaline and noradrenaline may be determined (35).

A different method of differentiation has been used by Manger *et al.* (21). It is based on the fact that the development of fluorescence from noradrenaline, but not from adrenaline, is inhibited by thiosulfate. The disadvantage of this method is that each sample must be divided into two aliquots, with the result that the sensitivity is halved.

Specificity

The relative fluorescence intensities given by a series of catechols in the ethylenediamine reaction under standard conditions and read through a yellow filter (Chance OY4) are shown in Table 1. The fluorescence formed from compounds with an acid function, and also from adrenalone, is extracted by isobutanol to only a slight extent; 3,4-dihydroxymandelic acid is an exception to this rule, probably because it has an hydroxyl group in the side chain and therefore loses its carboxyl group in the condensation reaction.

Two factors limit the number of interfering substances and the extent of their interference: 1) adsorption on alumina in column form and elution with a weak acid, and 2) the extraction of the fluorescent product with isobutanol. It has been shown by Drell (7) that catecholamines are eluted from the alumina column with acetate buffer pH 4 while catechol acids and 3,4-dihydroxyphenylalanine (dopa) require acetic acid for elution. Unfortunately the gain in specificity when the buffer is used for elution is obtained at the price of a tenfold increase in volume. On the other hand the small amount of acetic acid sufficient to elute the catecholamines in the standard procedure is insufficient for quantitative elution of 3,4-dihydroxyphenylacetic acid (dopac) or dopa. We find recoveries of about 60%. Combined with the unfavorable partition of the fluorescence in the isobutanol-water system, this factor reduces the interference caused by dopa and dopac to about 0.3 and 3% respectively, in relation to adrenaline. Both adsorption on a *column* of alumina, which gives sharper separation than batch adsorption, and isobutanol extraction of the fluorescent products are important steps in the

TABLE 2

*Fluorescence of catechol acids in ethylenediamine reaction after heating at 100°C for 20 minutes**

	Isobutanol Phase	Aqueous Phase
Dopac	11.2	10.2
3,4-dihydroxymandelic acid	16	1.6

* The values are relative to the fluorescence (designated as 100) of the isobutanol phase of an adrenaline standard, prepared at 50°C. Yellow filter (Chance OY 4).

method; to discard either as has been recommended (1, 10) is a step in the wrong direction.

The last traces of interference from acid catechols, including dopa, can be eliminated very easily by passing the eluate from the alumina column over a column of a weakly acidic cation exchange resin. In the final eluate adrenaline, noradrenaline and dopamine are, so far as we know, the only substances yielding fluorescence in the ethylenediamine reaction. This has been made the basis of a method for the estimation of dopamine which is determined as the difference of the fluorescence values found with the ethylenediamine method and those found with the trihydroxyindole method (37).

Catechol acids and dopa can be separated on a column of a strongly acidic cation exchange resin. The fraction of catechol acids will consist mainly of dopac and 3,4-dihydroxymandelic acid. In their quantitative estimation larger volumes of acetic acid or, preferably, sulfuric acid must be used for the elution of the alumina column. It is also advisable to carry out the condensation with ethylenediamine by heating at 100°C for 20 minutes. The two acids can readily be differentiated on the basis of the different partition of the condensation products between aqueous and isobutanol phases (Table 2).

Applications

Though the analysis of the acid fraction of catechols may yet become the more important application, the method has so far been used mainly for the analysis of the basic fraction. In combination with the trihydroxyindole method it offers a convenient way for the estimation of dopamine in extracts of urine and tissues (22, 37, 39).

The ethylenediamine method was originally proposed for the estimation of adrenaline and noradrenaline in plasma and evidence has been submitted in support of the validity of this application. Briefly, it was found that 1) the reacting material in plasma was alkali-labile and disappeared at the same rate as added adrenaline, 2) it was eliminated by a preparation of amine oxidase in a reaction which proceeded at the same speed and was inhibited by ephedrine to the same extent as that with added adrenaline, and which led to the complete disappearance of the reacting material, and 3) the plasma material was separated by paper chromatography into two components having the same R_f values and forming the same fluorescent derivatives as adrenaline and noradrenaline (34, 35, 36).

If dopamine were present in plasma, the estimates for adrenaline and noradrenaline obtained by the ethylenediamine method would be higher than those obtained by the trihydroxyindole method, since dopamine forms a fluorescent derivative in the former but not in the latter. In fact, the results obtained by the two methods were in good agreement (38). The absence of significant amounts of dopamine was also inferred from chromatographic evidence (38).

When the ethylenediamine method was applied to a plasma extract that had been purified only by alumina adsorption, the results were essentially the same as when it had, in addition, been adsorbed on a cation exchange resin; acids therefore did not significantly contribute to the fluorescence (38). This does not necessarily mean that no catechol acids occur in plasma. Von Euler (9) found considerable amounts of dopac in bovine plasma obtained from the slaughterhouse, but his procedure deviates in important details from ours: adsorption was carried out by the batch process and elution was effected with dilute sulfuric, instead of acetic, acid (11). Both of these modifications are likely to reduce the selectivity of the method and to increase the yield of catechol acids. Moreover, von Euler omitted the isobutanol extraction of the fluorescent condensation product which greatly reduces the interference from dopac. His conclusion that the presence of dopac in plasma renders the ethylenediamine method unspecific therefore ought to be qualified.

Although no evidence has been obtained for the presence of significant amounts of interfering substances in normal human plasma, it is likely that such material is encountered under pathological conditions, especially in cases of uremia (44). The trihydroxyindole method is not affected by these substances.

It may be asked if, in view of the undoubtedly superior specificity of the trihydroxyindole method, the use of the ethylenediamine method in blood analysis is still justified. Its main advantage, to my mind, is that a blank containing plasma extract becomes superfluous. This may seem surprising to those who regard the inclusion of such a blank as a particular virtue of the trihydroxyindole method. However, it has been shown that, when adrenaline and noradrenaline were removed from plasma by specific procedures, the ethylenediamine method yielded a fluorescence which did not exceed that of the reagent blank (18, 34). The complete content of catecholamines in the plasma sample is therefore available for conversion into fluorescent derivatives, an economy which is particularly important when consecutive blood samples are required from the same donor.

The results obtained with the ethylenediamine method in different laboratories show a considerable variation, but it may be pointed out that the discrepancies among workers using the trihydroxyindole method are just as great: while some find practically no adrenaline and a value for noradrenaline of about 0.3 μg/l (5, 27), others, such as Zileli *et al.* (44) and Munro and Robinson (23), like ourselves (38), find concentrations similar to those indicated by the ethylenediamine method.

As a partial explanation for these differences two factors in particular deserve attention: the activity of the subject and the choice of the anticoagulant. A basal state is probably approached only when the subject has been lying down

for a considerable time and all extraneous sensory stimuli have been reduced to a minimum. Some authors further recommend that the blood be taken through a cannula some time after it has been placed in the vein. We have never adopted these procedures because, quite apart from their inconvenience for clinical work, we felt that the concentrations of catecholamines circulating as a response to the normal stresses and activities of daily life, even if somewhat variable, would constitute a useful base line for many investigations.

With regard to the choice of anticoagulant, this is important since it affects the concentration of blood platelets in plasma and, inasmuch as platelets contain adrenaline and noradrenaline, indirectly also the concentration of adrenaline and noradrenaline in plasma (41). In particular it should be noted that, if heparinized blood is cooled in ice before centrifuging, extensive clumping of platelets occurs and the resulting plasma is practically platelet-free. This procedure will therefore give values which are 50 to 60% below those obtained on platelet-rich plasma. Similarly, excessive force and duration of centrifuging may lower the platelet content of plasma.

Unfortunately, these factors do not provide a complete explanation for the differences between the results of different laboratories. Table 3 shows the results of an experiment carried out in our laboratory in which the method of Cohen and Goldenberg (5) has been compared with the ethylenediamine method. Blood sample 1 was collected and processed exactly according to Cohen and Goldenberg and the final eluate analyzed by both fluorimetric methods. Blood sample 2, from the same donor, was collected in versene-thiosulfate solution (29) and the plasma freed from platelets by centrifuging. The platelet-poor plasma and the platelet fraction were purified according to our usual procedure (34, 41) and finally analyzed by the two fluorimetric methods.

The results obtained by the method of Cohen and Goldenberg agree reasonably well with those obtained by the ethylenediamine method whether they relate to

TABLE 3

*Comparison of method of Cohen and Goldenberg (C & G) with ethylenediamine (ED) method**

Plasma Sample	Plasma Fraction	Method	Plasma	
			Adrenaline	Noradrenaline
			μg/l	*μg/l*
1	—	C & G	0.37	1.94
		ED	0.60	1.46
2	Platelet-poor plasma	C & G	0.49	1.52
		ED	0.77	1.55
	Platelets	C & G	0.26	1.75
		ED	0.56	1.58

* *Blood sample 1* collected in heparin and cooled in ice before centrifuging. Plasma filtered through alumina column without previous dilution.

Blood sample 2, from same donor, collected in versene-thiosulfate solution. Plasma recentrifuged for 20 min at 3000 *g* to remove platelets. Platelet-poor plasma diluted with equal vol of 0.2 M sodium acetate, pH 8.4.

the plasma or the platelet fraction and whether the (platelet-poor) plasma has been prepared with heparin or with versene as anticoagulant. In this respect, therefore, the results of the method of Cohen and Goldenberg in no way differ from those previously obtained by us with other variants of the trihydroxyindole method (2, 36, 41). They are, however, quite different from those reported by Cohen and Goldenberg, although their method was followed in every detail, except that we used a different type of fluorimeter and a slightly different set of light filters.

It seems to me therefore that discrepancies such as these cannot be explained in terms of the different specificities of the ethylenediamine and trihydroxyindole methods, but are due to as yet unresolved differences in technique and instrumentation. It is to be hoped that agreement will be reached in due course. Meanwhile I should like to reaffirm my belief that the validity of the ethylenediamine method for the analysis of plasma rests on solid foundations although I am not shutting my eyes to the fact that it has not yet been possible to establish a satisfactory correlation with bioassays.

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