FATIGUE AND STRESS STUDIES:

An Improved Semi-Automated Procedure for Fluorometric Determination of Plasma Catecholamines

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FOREWORD

Stress may be regarded as an operational condition that challenges the self-regulating systems of man and demands a corrective response. In general, most of the stresses encountered during the course of ordinary life experiences are adequately compensated for and are usually of little concern. Those stresses that overwhelm man's compensating mechanisms are, however, often debilitating and result in generalized performance decrements.

The common denominator to many stresses is the response of the sympatho-adrenal system. Regardless of the origin of the stress, the application of the stressor is followed by a liberation of catecholamines (norepinephrine and epinephrine) from the sympathetic nerve endings and from the adrenal medulla. The present report describes a new application of the chemical technique for the estimation of catecholamines in blood. Such measurements may permit a quantitative evaluation of the magnitude of stresses encountered in specific work situations and may provide an index to man's tolerance for various specific stress exposures.

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I. Introduction.

The use of an automated chemical analytical system (Autoanalyzer, Technicon Instruments Corporation, Chauncey, New York) as an adjuvant in routine assay for catecholamines has been explored by Merrills [1, 2] with promising results. Based on a fluorometric trihydroxyindole (THI) method,[3] the procedure uses to best advantage the constant rates of sample and reagent introduction and the fixed durations for chemical reaction provided by the Autoanalyzer. Although the method described is useful for estimating catecholamines in tissues or fluids containing relatively high concentrations of those compounds, it unfortunately lacks sufficient sensitivity for the determination of catecholamines in human peripheral plasma.

The present report details an improved semi-automated procedure based on Merrills' original suggestion. Several modifications in both the isolation and detection steps of the method have been introduced in order to achieve the sensitivity and specificity required for the estimation of the low levels of catecholamines generally found in resting plasma.

II. Materials and Methods.

A. *Reagents.*—All chemicals used were reagent-grade or highest-purity materials. Solutions were prepared with twice-distilled water, the final distillation from an all-glass apparatus.

1. Alumina (Woelm), neutral, activity grade 1 for chromatography. Prior to use, the alumina was purified according to the procedure described by Anton and Sayre.[4]
2. Sodium acetate, 0.2 M. This solution was purified by passage through a 25- by 300-mm column of Chelex 100 (Bio-Rad Laboratories, Richmond, California), 50-100 mesh, Na^+ form. The pH of the purified buffer was adjusted to 8.4±0.1 (glass electrode) with 0.5 N sodium carbonate.
3. Ethylenediaminetetracetate disodium, 5% (w/v).
4. Acetic acid, 0.3 M.
5. Sodium acetate, 1.5 M, containing 0.01% (w/v) potassium ferricyanide. This solution was passed through a medium-porosity sintered-glass filter. The pH of this reagent as prepared is 9.0 ±0.1.
6. Ascorbic acid, 0.3% (w/v). The Eastman product was used without further purification.
7. Sodium hydroxide, 2.5 M.
8. Perchloric acid, 4 N.

B. *Standards.*—Stock standard A, 1.0 mg/ml total catecholamines as the free bases, was prepared from dl-arterenol hydrochloride and l-epinephrine bitartrate (Winthrop Laboratories, New York, New York) in the ratio of 2:1 in order to simulate that ratio found in human peripheral plasma.[4] Stock standard B, 10 μg/ml, was prepared from A; stock standard C, 1 μg/ml, from B. Working standards containing 1 to 5 μg/*l* total catecholamines were prepared from stock standard C. All standards were prepared in a stabilizing solution of 0.01 N HCl containing 0.5 mg/ml sodium metabisulfite. Stock standards A and B, stored under refrigeration, are stable for at least 1 month. Stock standard C was prepared weekly; working standards were freshly prepared before each analysis.

C. *Apparatus.*—

1. Adsorption tubes found most convenient had the following dimensions: reservoir, 90 by 25 mm (ID); stem, 17 by 4 mm (ID). A pipette tip (1 mm) was drawn from the stem to facilitate draining. Reservoir capacity was 40 ml. Ten such tubes can be handled simultaneously by incorp-

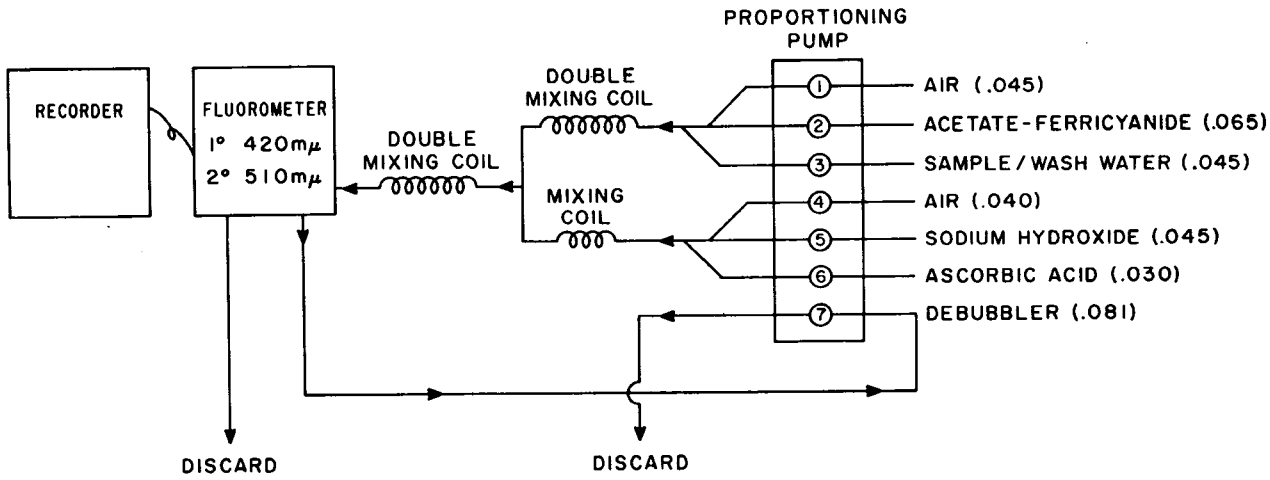

FIGURE 1. Flow diagram for automated fluorometric catecholamine assay. Figures in parentheses are inside diameters of pumping tubes. Model II sampler module was used in the system described. A standard sampler module can be incorporated if lines 1 and 3 are attached to a double-crook sampling device to maintain a fixed fluid: air ratio in the system. Samples are processed at the rate of 20/hour.

orating them in a negative-pressure manifold. Uniformity of flow from tube to tube was maintained by individual stopcock control.

2. Automatic analysis. A standard Autoanalyzer train (sampler, proportioning pump, and recorder), coupled with a Turner fluorometer (Model 111), provided the automatic system for catecholamine oxidation, alkaline rearrangement, fluorescence detection, and recording. A schematic flow diagram for the system is shown in Figure 1. Through the use of a Sampler II Module, a constant air: fluid ratio was maintained within the system, minimizing mixing variations and permitting efficient debubbling at the flow cuvet. Ferricyanide oxidation and alkaline rearrangement occurred in separate double-mixing coils, permitting reaction times of 100 seconds for each step.

To increase the sensitivity of the detection system, a high-sensitivity unit (Turner) was adapted to permit use of a standard fluorescence flow cuvet (Technicon). The adapter consisted of a cylindrical aluminum sleeve, 72 mm long, 12 mm in OD, and 8 mm in ID. Four slits were milled into the sleeve (25 by 3 mm) at right angles to each other. This adapter permitted the use of a 30X light intensity. Excitation light at 420 mμ was providing by combining a Wratten 2C gelatin and a 3-mm-thick Schott BG-3. A secondary filter combination of Wratten 8 and Wratten 65A provided emission light peaking at 510 mμ. A continuous-emission (400 to 520 mμ blue lamp (T-5 envelope) replaced the general-purpose lamp normally supplied with the Turner fluorometer.

D. *Procedure.*—Portions of blood (25 ml), drawn in syringes lubricated with heparin, were transferred to 40-ml centrifuge tubes containing 5 mg of sodium metabisulfite. The tubes were chilled in shaved ice and centrifuged at 3,000 rpm for 15 minutes. Plasma was separated and stored at −20°C for analysis.

One milliliter of 4 N perchloric acid was added to each 10.0 ml aliquot of plasma. The mixture was agitated thoroughly and centrifuged at 30,000g for 10 minutes at 10°C to remove all protein. Aliquots (9 ml) of the protein-free supernatant solution were added to 50-ml beakers containing 10 ml of 0.2 M sodium acetate, 1.0 ml of 5% EDTA, and 500 mg of acid-washed alumina. Stabilizing solution, treated as sample and carried through the entire procedure, served as the column (reagent) blank. The pH of the mixture was adjusted to 8.4 ±0.1 (glass electrode) and maintained at that pH for 4 minutes (timed) by the dropwise addition of 5.0 N sodium carbonate. The mixture must be agitated continually during this adjustment in order to distribute the particles of alumina evenly throughout the solution. A motorized glass stirring rod and a small turntable (10 rpm) to rotate the beaker

worked satisfactorily. At the end of the 4-minute period, the alumina was transferred as a slurry to the adsorption tubes. The alumina rapidly settled as an air-free column. The solutions above the alumina were then drawn through the columns at the rate of 1 to 1.5 ml/min. Columns were next washed with 40-ml glass-distilled water (pH 8.4 ±0.1) at a flow between 1.5 and 2 ml/min. As the final step, the alumina was eluted with 2.0 ml of 0.3 M acetic acid, and the eluates were centrifuged at 30,000g (10°C) to precipitate the fine suspension of alumina occasionally encountered after elution.

Centrifuged eluates were transferred to standard sample cups and processed through the Autoanalyzer at 20 samples/hour. Peaks of unknown samples were compared with those of standards run through the entire procedure simultaneously.

All phases of the procedure were conducted under red illumination (45 foot-candles).

III. Results.

Over the range to 5 $\mu g/l$ original standard, fluorescence intensity is linearly related to total catecholamine concentration under the conditions of the procedure described here. Figure 2 shows the standard curve derived from 10 complete determinations with a measure of the variability (SD) at each concentration. A typical recording, from the Autoanalyzer system, of fluorescence intensity as a function of concentration is shown in Figure 3.

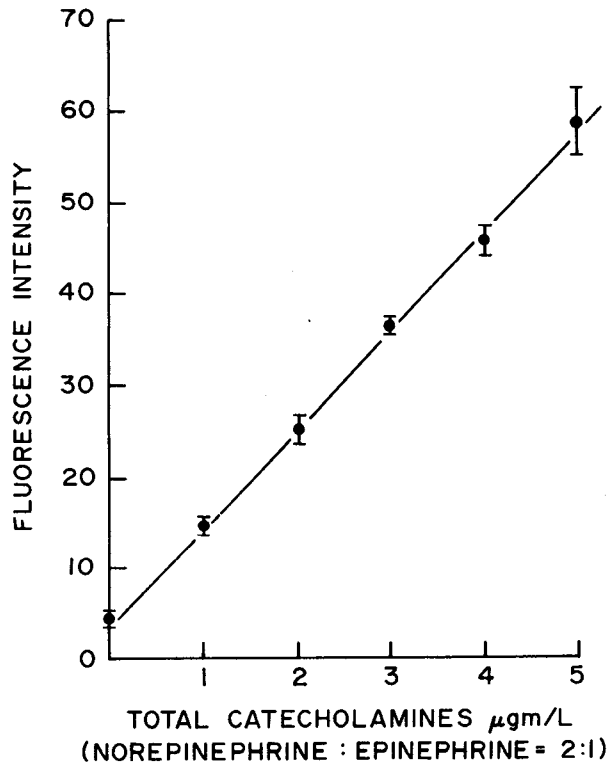

FIGURE 2. Standard curve for total catecholamine concentration through 5 $\mu g/l$. Each point represents mean fluorescence of 10 determinations. Vertical lines are the standard deviation at each concentration.

Two types of recovery studies were performed to establish the accuracy of the procedure. Efficiencies of alumina adsorption and elution were determined by carrying 10.0 ml of standard (1 or 5 $\mu g/l$) through the perchloric acidification and adsorption steps. Columns were eluted with 2.0 ml of 0.3 M acetic acid, and the eluates were diluted back to 10.0 ml with 0.3 M acetic acid, pH 3.85 (mean pH of acid eluates).

Fluorescence of diluted samples was compared to corresponding concentrations of standard in 0.3 M acetic acid, pH 3.85. Ratio of fluorescence, alumina-treated: direct, is a measure of the efficiency of isolation. Recoveries from plasma were determined by adding to *plasma* amounts of total catecholamines corresponding to quantities likely to be estimated (0.01 or 0.02 μg/10.0 ml of plasma). The ratio between the fluorescence increment of plasma with added catecholamines and a corresponding standard represents the efficiency of extraction of the amines from plasma. Recovery data are summarized in the Table.

TABLE. Recovery of Total Catecholamines from Aqueous Solutions and from Plasma.

Quantity of total catecholamines added to 500 mg alumina	Number of determinations	Recovery of added catecholamines
μg		% ±SD
0.01 in 10.0 ml of stabilizing solution	9	92.5 ±11.1
0.05 in 10.0 ml of stabilizing solution	9	74.8 ±5.1
0.01 in 10.0 ml of human plasma	9	86.4 ±8.9
0.02 in 10.0 ml of human plasma	9	85.6 ±5.8

Considering the nearly fivefold concentration of catecholamines provided by adsorption from 9.0 ml of original sample and elution with 2.0 ml of acid, 50 units of deflection (based on a scale of

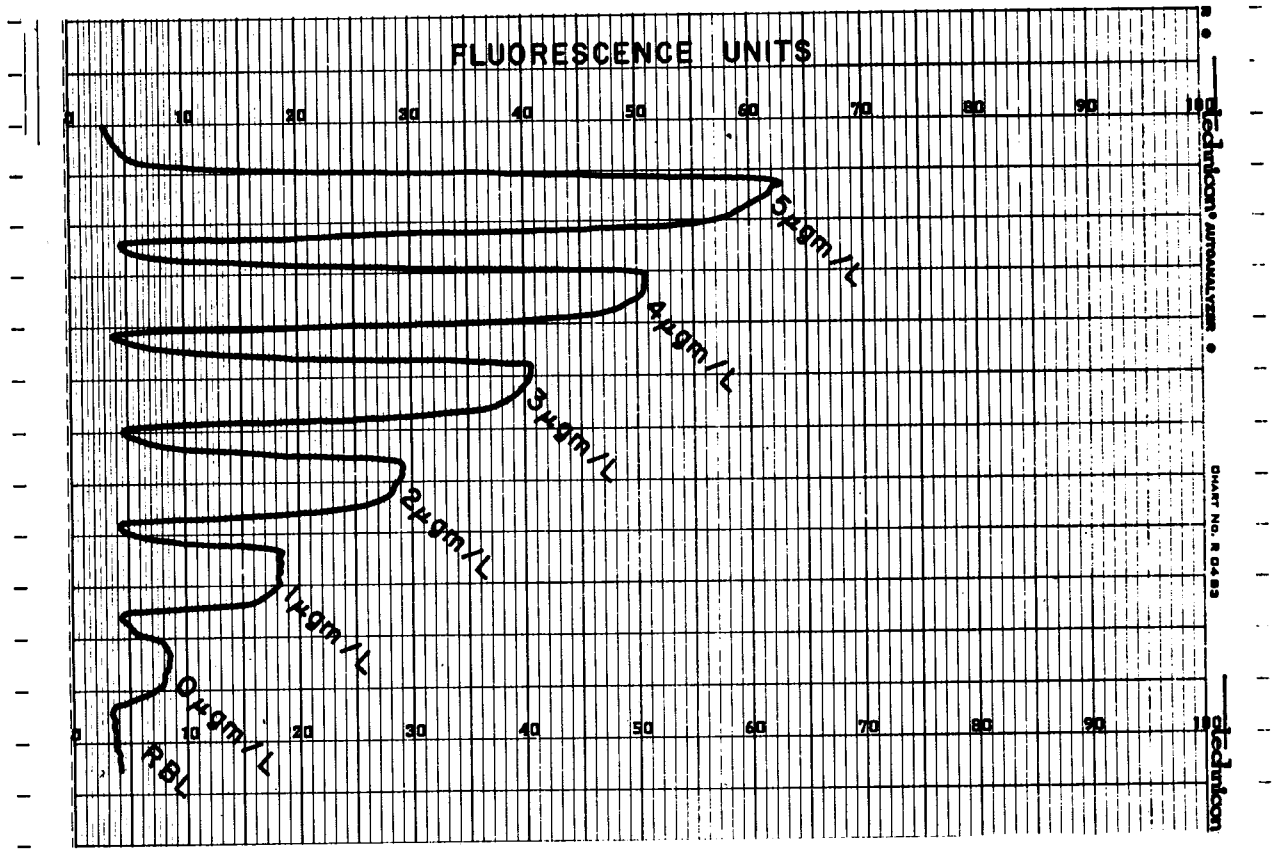

FIGURE 3. Recorder tracing of five standards carried through the entire procedure. Column blank represents 0 $\mu g/l$. *RBL* is the reagent baseline level. Concentrations are are in terms of total catecholamines (free bases), norepinephrine : epinephrine =2 :1.

100) were obtained with original solutions containing approximately 4 $\mu g/l$. Given this sensitivity, total catecholamines can be readily estimated from 1.5 to 2.0 ml of acid eluate containing as little as 0.0020 μg/ml of the amines.

Resting levels of total catecholamines in plasma were determined in 20 male subjects, 20 to 29 years old. Subjects were postabsorptive and remained at bedrest for 30 minutes prior to sampling. The mean resting concentration of total catecholamines, determined from analysis of 10.0-ml aliquots of plasma, was 0.89 $\pm$0.15 (SD) $\mu g/l$.

IV. Discussion.

Application of the principles of automated chemical analysis in the estimation of plasma catecholamines provides the immediate advantage of reproducibility in two critical steps of the THI assay: (a) the time during which the catecholamines are oxidized by ferricyanide and (b) the duration of alkaline rearrangement of adrenochrome and noradrenochrome to the corresponding fluorescent lutines. The importance of rigid standardization of these reaction times has been emphasized by several authors.[4-6] In the system described here, reaction times are determined solely by the lengths of the mixing coils in which those reactions occur, since flow in those channels is maintained constant. Once appropriate coil lengths have been determined, these steps remain unvarying from sample to sample and require no further attention. Introduction of the ferricyanide reagent together with buffer also allows automatic pH adjustment of the acid eluate. In this system, the 0.3 M acetic acid eluate has a final pH of 3.85 $\pm$0.05. Mixing eluate (sample) and buffered ferricyanide in the molar strength and ratio designated yields an oxidation mixture of pH 5.8. This pH is favorable for the oxidation of both epinephrine and norepinephrine.

Incorporation of the dialyzer module as described by Merrills [1] is an unnecessary refine-

ment if fluorescent contaminants do not appear in the final sample. By obviating the use of an internal pH indicator, the present procedure excludes the need for dialysis and the attendant loss of about 75% of the catecholamine activity initially present in the eluate.

Isolation of catecholamines by adsorption from plasma onto alumina is a common step in both the THI and ethylenediamine conjugation procedures. Experience in this laboratory has shown that performing the adsorption from plasma as generally described [5,7,8] yields an eluate that is not devoid of protein (as indicated by the formation of a fine precipitate after addition of 4 N perchloric acid). Fluorescence intensities obtained after direct adsorption from plasma are approximately 2.5 times higher than those secured by making the adsorption from a protein-free supernate of plasma. These results indicate that traces of plasma protein must also be adsorbed onto alumina in quantities sufficient to interfere with fluorescence measurement of the catecholamines. Prior removal of protein with perchloric acid avoids this difficulty.

Although column chromatography is considered a more efficient process than "batch" adsorption,[9,10] the present modifications include a combination of the two. Thorough mixing of alumina, sample, and buffer during pH adjustment, followed by a column step, resulted in column recoveries of greater than 90% when physiological quantities of both amines were present. At higher concentrations (5 $\mu g/l$), column recoveries averaged around 75%, indicating some reduction in the efficiency of either adsorption or elution at this concentration. Prior experiments with solutions containing only epinephrine indicated that column adsorption alone was sufficient to extract the amine efficiently. If both amines were present in the ratio of 2NE:1E, however, a reduction in senistivity of approximately 30% resulted if only column adsorption was used (Figure 4). Increased sensitivity was achieved with the combined standard by using the combination procedure finally adopted. Such data suggest that the rates of adsorption of the two amines may not be similar under the conditions described although the adsorption of both is optimized in the present procedure.

Recoveries of catecholamines added to human plasma *before* protein precipitation in quantities equivalent to 1 or 2 $\mu g/l$ (0.01 μg/10 ml or 0.02

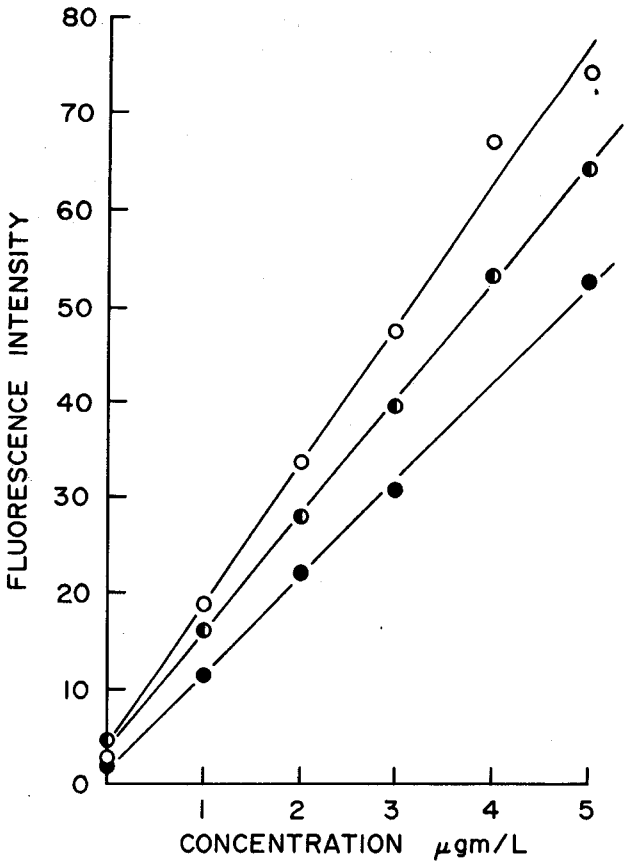

FIGURE 4. Sensitivity of the assay (slope) as a function of the amines present in the original standard and the mode of adsorption. Epinephrine, column adsorption only, 0–0; norepinephrine-epinephrine mixture (2:1), column adsorption only, ●–●; norepinephrine-epinephrine mixture (2:1), batch plus column adsorption, ◐–◐.

μg/10 ml) averaged 86.4% and 85.6%, respectively.

The value for resting concentration of plasma total catecholamines determined by this procedure is lower than that reported by Anton and Sayre (E + NE)[4] although both are somewhat higher than others obtained with the THI procedure.[5,6,11] The reason for these differences is not immediately apparent, although the higher value of Anton and Sayre may in part reflect the sampling situation. It is interesting to note, however, that in none of the other THI procedures cited [5,6,11] is an antioxidant added to plasma prior to adsorption. In both this method and that of Anton and Sayre,[4] sodium metabisulfite is routinely added to blood immediately after sampling. The presence of this reducing material in the

plasma sample (and in the catecholamine standard in the current procedure) insures its availability during the pH adjustment for optimal alumina adsorption (8.4). At this pH, without protection, catecholamines are highly susceptible to oxidative destruction. The influence of added antioxidant on retarding the loss of catecholamines during assay of biological fluids has been discussed by Anton and Sayre,[4] Weil-Malherbe,[10] and Manger.[12] Whether this point, in itself, is sufficient to explain the lower values reported for total catecholamines in normal plasma cannot be presently decided.

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