ARTERIOVENOUS DIFFERENCES OF THE NOREPINEPRINE-LIKE MATERIAL FROM NORMAL PLASMA AND INFUSED NOREPINEPHRINE *

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All physiological stimuli result in changes in the level of activity of the autonomic nervous system. A method of monitoring the over-all activity of the adrenergic nerves and adrenal medullae would greatly simplify the interpretation of many physiological events. The present work began with the assumption that blood levels of norepinephrine (NE) and epinephrine (E) might reflect adrenergic activity. Therefore, our initial objective was to modify one of the existing chemical methods for the measurement of NE and E so that it would be suitable for our purposes. However, we became impressed with the controversy in the literature over the chemical identification of plasma NE and E and, as a result, adopted the dual criteria that plasma material must have not only the chemical properties of NE and E but must be removed by the tissues like NE and E to justify identification as these substances.

The trihydroxyindole (THI) method of Lund (1–4) and the ethylenediamine (EDA) method of Weil-Malherbe and Bone (5–7) are the two methods currently considered suitable for analysis of plasma NE and E. Neither method is specific. In this respect, the EDA method is inferior to the THI method (6, 8, 9), a fact which is emphasized by the false high readings of the former in uremia (10). However, the THI method may measure dopamine (3, 4), dihydroxyphenylalanine (2–4), isopropylnorepinephrine (2–4, 9), serotonin (2) or aureomycin (11), depending on the modification used. To a greater (EDA method) or lesser degree (THI method), the proponents of both methods have depended upon the fact that none of the possibly interfering analogs of NE and E has as yet been demonstrated in normal blood by fluorometry, paper chromatography or bioassay. Cohen and Goldenberg (3) cited the results of infusion experiments as important evidence that their method (THI) did not measure the metabolites of NE or E. Steady infusions produced steady blood levels, which would not be expected if accumulating metabolites were also determined. We have had a similar experience with the EDA method.

The difficulty in preparing reagent blanks which fluoresce with the same intensity each day indicates that contaminants are capable of producing fluorescence which may be interpreted as NE and E. Both methods are subject to this difficulty. In our opinion, this type of nonspecificity has interfered more with the quantitative aspects of the methods than has nonspecificity due to possible interference by the metabolites of NE and E or other closely related compounds.

Whether or not either method as reported to date can accurately measure known NE and E in the amounts said to be present in normal plasma is also open to question. Price and Price (2), using the THI method, reported normal venous levels of 0.01 μg E per L and 0.34 μg NE per L. They used 15-ml samples of plasma but gave no data on their ability to distinguish unmodified plasma from plasma with added E and NE in amounts of 0.00015 μg E per 15 ml or of 0.0051 μg NE per 15 ml. They stated that differences in replicates of a single sample varied as much as did recoveries of small amounts of added material. They noted that their faded blanks varied in proportion to the amounts of E or NE which had gone into making them up. Cohen and Golden-

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TABLE I

Mean galvanometer deflections per 0.001 μg of NE or E added to 5-ml aliquots of plasma *

	Blue-green fluorescence	Yellow fluorescence
NE	5.7 (SD ± 1.1)	3.8 (SD ± 0.5)
E	1.6 (SD ± 1.0)	12.0 (SD ± 1.9)

* The actual measurements from which the table is derived were made on 9 different plasmas. Epinephrine in 2 concentrations (0.002 μg per 5 ml and 0.004 μg per 5 ml) and norepinephrine in 2 concentrations (0.004 μg per 5 ml and 0.008 μg per 5 ml) were determined in duplicate in each plasma.

berg (3) also used 15-ml plasma samples and reported normal levels of 0.06 μg E per L and 0.30 μg NE per L. They also gave no data on the difference between unmodified plasma and plasma with added E and NE in amounts of 0.0009 μg E per 15 ml or of 0.0045 μg NE per ml. They stated that "the small amounts of fluorescence above blanks detected in normal plasma specimens had associated with them a low degree of precision and reliability."

Vendsalu (4), using the THI method with samples of 10 ml, found normal levels of 0.07 μg E per L and 0.35 μg NE per L. He reported a recovery of 83 per cent of E and NE added to plasma in amounts which were, on the average, higher than these concentrations. In discussing normal plasma values, he remarked that the limit of sensitivity was governed by the blank and that resolution was diminished when the height of the sample peak was low and near to the blank. These problems have been given little attention in recent EDA papers (6–8, 12, 13).

None of the authors referred to above explains why he uses standard solutions of E and NE which are many times more concentrated than normal plasma. In our experience, instrument settings based on such standards are too insensitive for quantitative work with NE or E added to plasma in apparently normal concentrations.

We elected to explore further the potentialities of the EDA method because of greater blank stability at high sensitivities (14) and because the final extraction into isobutanol afforded the opportunity of effecting an increased concentration of the desired materials over the blank.

This paper will present data on four points: *1*) modifications of the EDA method which make it more quantitative at low concentrations of NE and E; *2*) the concentration of material in normal plasma with the fluorescent characteristics of NE and E; *3*) arteriovenous (A-V) differences of the material found in normal plasma which was assumed to be NE; and *4*) A-V differences of infused NE.

METHOD

The method of Weil-Malherbe and Bone (5), as modified by Aronow and Howard (15) and further modified by ourselves, is used. The blanks and samples are made stable and reproducible by scrupulous attention to sources of contamination, such as the glassware, groundglass stoppers, room dust, tobacco smoke, impurities in reagents, and so forth. The volume of isobutyl alcohol used in the final extraction is 0.5 ml. This is read in a 6 × 6 mm square Pyrex cuvet in a Farrand model A fluorometer. The exciting light is the 436 mμ mercury line, and fluorescence is read at 485 mμ and above 530 mμ. The fluorometer-galvanometer unit is set at the same sensitivity daily by means of fluorescent filter glass. The sensitivity selected was that which gave readings for normal plasma near the center of the galvanometer. Standard solutions read near the top of the galvanometer at this setting.

Heparin was found to be a source of variability and was replaced by ethylenediamine tetraacetic acid (EDTA) as the anticoagulant. Blood samples cooled in ice water are stable for at least 90 minutes. Thiosulfate preservative was extensively investigated and found to be unnecessary. All samples, blanks and standards are run in duplicate. Five-ml samples of plasma and 125 mg alumina are used.

RESULTS

A. Sensitivity and accuracy. Standards containing 0.002 and 0.004 μg of E and 0.004 and 0.008 μg of NE per 5 ml of plasma were used to determine the number of galvanometer deflections given by NE and E with the two sets of filters. The reading of 485 mμ (blue-green filter) indicates primarily NE and that above 530 mμ (yellow filter) indicates primarily E. The average value given by the standards over a period of days was used in calculating the NE and E content of plasmas containing unknown amounts of NE and E. This allowed us to calculate the deviation of the standard from the mean (Table I). As the concentrations of NE and E in the standards and unknowns were of the same order of magnitude, losses in the procedure were equal in the two groups and the mean recovery in a series of known solutions approached 100 per cent (Table II). Changes in batches of reagents or a new

light source or photomultiplier tube in the fluorometer affected the number of galvanometer deflections per unit of material and required the establishment of new sets of values.

Table II gives the recovery of mixtures of standard amounts of NE and E. Comparison of these data with the normal values given below clearly shows that this method is capable of the quantitative recovery of added NE in the apparent normal range and that physiological concentrations of known materials can be used as the regular standards. The amount of material in normal plasma giving the fluorescent pattern of E is too small for accurate quantitation in a single determination. In a series of determinations, plasma with E added in this concentration can be separated from plasma without added E.

Standards similar to those reported in Table I can be run each day or each week. If the sensitivity of the fluorometer-galvanometer unit is kept constant by the use of the fluorescent glass filter, and if the reagent blanks are constant, weekly standards are sufficient.

B. Normal values for material in plasma measured as NE and E. The average arterial and venous concentrations of apparent NE and E found in normal university men are given in Table III. All samples were obtained through indwelling needles from subjects lying quietly in bed. These values are lower than those previously reported with the EDA method (5–10, 13, 16, 17). In our opinion, the values are lower than those usually reported for the EDA method because of the elimination of many fluorescent contaminants. The values for NE are 100 per cent higher than

TABLE II

Calculated mean amounts of NE and E added as a mixture to 5-ml aliquots of plasma on 9 different days *

Amounts of NE and E actually added to 5 ml plasma		Amounts of NE and E calculated to be in the mixture	
	ug		*ug*
NE	0.004	NE	0.0037 (SD ± 0.0005)
E	0.002	E	0.0018 (SD ± 0.0002)

* The measurements were made on the same days as those in Table I. The calculations are based on the observed galvanometer readings with the blue-green and yellow filters from the aliquots containing only NE or E (Table I). Because of the experimental design, it is assumed that recoveries would approach 100% if the series were expanded indefinitely.

TABLE III

Arterial and venous concentrations of the material measured as NE in normal subjects

	Mean	SD
	μg/5 ml plasma	
A. 47 Subjects, simultaneous arterial-femoral venous samples		
Artery*	0.0036	±0.0018
Vein	0.0040	±0.0015
B. 7 Subjects, 19 simultaneous arterial-antecubital venous samples		
Artery*	0.0037	±0.0017
Vein	0.0040	±0.0019

* The difference between arterial and venous concentrations is not significant, $p > 0.05$. The difference becomes significant when the 47 determinations in A are combined with the 19 determinations in B and analyzed together.

those reported with the THI method (2–4). A part of this difference is predictable, as our experimental design allowed approximately 100 per cent recovery of added material, and the THI method supposedly recovers about 80 per cent of added material. This accounts for 40 per cent of the difference. Data which allow detailed analysis of recoveries with the THI method at the levels of concentration under discussion are not available. This makes interpretation of the significance of the remainder of the difference uncertain.

C. Is there an arteriovenous difference of the material measured as NE and E? Using a modified THI method, Vendsalu (4) reported that the mean A-V differences across an extremity in 29 healthy subjects were 0.16 μg E and − 0.09 μg NE per L. To be certain that our method was accurate enough to measure differences in these ranges, five sets of observations were made on five different days. Each day pooled plasma was divided into three aliquots. To one aliquot was added 0.00045 μg NE per 5 ml, to the second 0.0008 μg E per 5 ml, and nothing was added to the third. Thirty-four comparisons were made between the unmodified plasma and either of the other two aliquots. The technician working with the samples did not know the purpose of the experiment and did not know that half of the samples contained unmodified plasma. Twenty-four comparisons were made between plasma and plasma plus NE. There was enough spread in the readings so that the 12 highest plasma samples could not be distinguished from the 12 lowest

TABLE IV

*Arterial-antecubital venous differences of the material measured as NE and E in 7 subjects with and without occlusion of the wrist**

	Wrist unoccluded			Wrist occluded		
	Mean	SE	Significance of difference	Mean	SE	Significance of difference
	μg/5 ml plasma			*μg/5 ml plasma*		
NE	−0.0004	±0.0002	$p > 0.05$	−0.0004	±0.0003	$p > 0.10$
E	+0.0002	±0.00009	$p < 0.025$	+0.0002	±0.00007	$p < 0.005$

* The data were obtained from 19 A-V pairs with the wrist unoccluded and 19 pairs with the wrist occluded.

samples of plasma plus NE. All of the remaining 12 NE samples were higher than any of the remaining 12 plasma samples. Statistical analysis of all 24 comparisons showed the aliquots to be different ($p < 0.01$), the calculated mean differences having been 0.0007 ± 0.0006 μg NE per 5 ml. Actually, only 12 *random* comparisons of the two aliquots were necessary to demonstrate the difference (p 0.02). Although this method cannot *quantitate* differences in plasma concentration of 0.00045 μg NE per 5 ml, it does reliably identify such a difference in as few as 12 comparisons. It cannot identify this difference in a single comparison.

All ten samples of plasma plus E were higher than any of the ten plasma samples with which they were compared. The calculated mean difference was 0.0007 ± 0.0002 μg E per 5 ml. To our knowledge similar data have not been offered in support of the ability of any other method to measure differences in plasma concentrations of added NE and E in the range of apparent peripheral arteriovenous differences.

In view of the ability of this method to identify such differences in added NE and E in a relatively few comparisons, re-examination of the data in Table III is of interest. The small negative A-V difference of the material called NE (0.0004 μg per 5 ml plasma) observed across the leg in 47 subjects was not significant. Neither was the same difference noted across the arm in 19 samples from 7 subjects, the p value having been greater than 0.05 in both cases. If these data are combined, the A-V difference does become significant, p falling to less than 0.01. It appears that there is, on the average, a small negative A-V difference of the material called NE but that the arterial and venous concentrations are too similar in many people to permit its demonstration except in a sizable population.

The small positive A-V difference of the material called E (0.0002 μg per 5 ml) is significant. Interpretation of epinephrine data will not be included in this paper.

As noted above, some workers (2, 4), including ourselves at an earlier date (18), found higher levels of NE-like fluorescent material in venous blood than in arterial blood. It has been assumed that the venous material was true NE produced by the adrenergic nerves in the hand and forearm. In an attempt to clarify this point, indwelling needles were placed in the brachial artery and antecubital vein of seven normal men lying quietly in bed. Every 10 minutes, arterial and venous samples were drawn. For 3 minutes preceding each alternate drawing, a cuff at the wrist was inflated to 250 mm Hg. Data are given in Table IV. The mean A-V difference for the material called NE was negative, but not significantly so, and was unchanged by wrist occlusion. As was expected, venous blood in the samples collected with the wrist occluded contained less oxygen than did those without the occlusion.

These data are of interest because we expected the venous blood draining the hand to be a favorable site for the demonstration of NE. The arterial blood passing through A-V communications would have lost little NE in its passage through the tissues. The hand is well supplied with adrenergic nerves and, under the conditions of this experiment, continuous autonomic nervous activity is likely to be present. The addition of blood from areas where NE is made in the hand to a stream of venous blood from which no NE had been lost creates a situation which would appear favorable for the demonstration of the addition of NE to venous blood. However, the observed small negative A-V difference of the NE-like material (− 0.0004 μg per 5 ml) was not significant

in the normal arm and was not further narrowed by exclusion of the hand from the circulation.

D. Do the endogenous material called NE and infused NE behave similarly in the blood stream? As pointed out above, no reported fluorometric method is entirely specific for NE and E. Both the THI and EDA methods are presumed to measure only NE and E in normal peripheral plasma. It appeared desirable to determine whether the material in plasma presumed to be NE was treated by the tissues in the same way as infused NE.

Nineteen normal subjects ranging in weight from 113 to 57 kg (mean, 80 kg) were given infusions of NE into an antecubital vein at the constant rate of 4 μg per minute (average, 0.05 μg per kg per minute). The average increase in blood pressure was 14/10 mm Hg and the average decrease in pulse rate was 8 beats per minute. Most subjects had no symptoms. Simultaneous blood samples from a brachial artery and a femoral vein were drawn before infusion and after at least 10 minutes of infusion. Table V summarizes the data. Concentration of the endogenous material resembling NE was greater in the artery in 8 subjects and greater in the vein in 11 subjects. The arterial and venous concentrations were not significantly different. The average concentration of the NE in arterial plasma produced by the infusion was twice the concentration of NE-like material present before infusion. Arterial levels from the infused material were higher than venous levels in all subjects, averaging nearly four times the femoral venous concentrations. In three subjects, no infused NE appeared in the femoral vein, removal in the leg having been complete. On the average, the leg removed 72 per cent of the arterial NE. In four subjects, antecubital venous samples (side opposite the infusion) were also measured and showed in each instance a positive A-V difference of infused NE. These data clearly demonstrate the efficient removal of arterial NE by the leg. By contrast, there was not a significant femoral A-V difference of the material measured as NE in resting plasma.

TABLE V

Femoral A-V differences of infused NE and the plasma material measured as NE in 19 subjects

	Plasma material measured as NE		Infused NE	
	Mean	SD	Mean	SD
	μg/5 ml plasma		*μg/5ml plasma*	
Artery	0.0039*	±0.0015	0.0081†	±0.0031
Vein	0.0045	±0.0011	0.0023	±0.0022

* The A-V difference is not significant, $p > 0.05$.
† The A-V difference is significant, $p < 0.001$. The values of infused NE are derived by subtracting the fluorescence of the appropriate control samples from the total fluorescence of the samples obtained during infusion.

TABLE VI

Hepatic A-V differences of infused NE and the plasma material measured as NE

	Plasma material measured as NE in 14 subjects		Infused NE in 5 subjects*	
	Mean	SD	Mean	Range
Artery	0.0036†	±0.0020	0.0220	0.0132–0.0280
Vein	0.0020	±0.0013	0.0013	0.0002–0.0035

* Infusions ranged from 7 to 26 μg NE per minute.
† The A-V difference is significant, $p < 0.01$.

Arteriovenous differences across the liver were also compared. The subjects were 14 patients who underwent diagnostic right heart catheterization for valvular heart disease. None had clinically evident liver disease. Liver function tests were either normal or mildly abnormal in a pattern consistent with mild congestive heart failure. Data are summarized in Table VI. Values for the NE-like material were only 55 per cent as high in hepatic venous plasma as in simultaneously sampled arterial plasma. Appreciable concentrations (average, 0.0020 μg per 5 ml) of the NE-like material remained in the hepatic venous blood of most patients. Five patients were given infusions of NE into an antecubital vein at rates varying from 7.0 to 26.0 μg per minute, the average rate having been 14.2 μg per minute. Pronounced elevations in arterial NE occurred in every patient but elevations in hepatic venous blood were much lower, averaging only 6 per cent of the arterial level. The average level of infused NE in the hepatic venous blood was less than the level of the NE-like material present before the infusion.

The endogenous material and infused NE were different in the following respects: *1*) 94 per cent of the infused NE but only 45 per cent of the endogenous NE-like material was removed in the liver; *2*) 72 per cent of the infused NE and none

of the endogenous material was removed in the leg.

DISCUSSION

Material forming fluorescent products with EDA having the pattern of those formed by NE is present in normal arterial plasma in an average concentration of 0.72 μg per L (0.0036 μg per 5 ml). When NE is infused intravenously at the rate of 4 μg per minute, the average concentration of material in arterial blood having the fluorescent pattern of NE is increased by 200 per cent; the average concentration in venous blood is increased by 50 per cent. The NE-like material in normal arterial plasma has an A-V difference across the extremity of − 0.08 μg per L (− 0.0004 μg per 5 ml). This difference requires over 47 determinations to reach a significant p value. The NE added by the infusion always has a positive A-V difference, and in some instances none of it appears in femoral venous blood. The splanchnic A-V difference for infused NE is greater than that for the material in normal plasma.

The material in normal plasma having the fluorescent characteristics of NE is present in equal amounts in blood from the femoral and antecubital veins. At low levels of infusion the concentration of NE in femoral venous blood is less than in antecubital venous blood. The concentration of the material in normal plasma is the same in blood draining the forearm and hand as in blood draining only the forearm.

These observations raise the question of whether there is a measurable quantity of NE in arterial and venous blood. At a concentration of 0.72 μg per L most of the arterial NE would be removed in the leg and arm. If the material thought to be NE is indeed NE, it appears reasonable to assume that NE is removed from the arteriolar and capillary bed in one area and added back to the blood by diffusion from the nerve endings of the autonomic nervous system at a site distal to the area of removal. In normal subjects the amount removed and the amount added would appear to be in remarkably close balance, since an A-V difference of NE-like material is not demonstrable in many subjects, although a small difference can be shown in a large series. Furthermore, the rates of removal and addition would seem to be such that the venous blood from such diverse tissues as the forearm and hand contained the same concentration of NE.

It has generally been assumed that the NE found in the urine after removal of the adrenal medullae comes from NE made at adrenergic nerve endings. A further assumption has been that the NE reached the kidneys by way of the blood. There is no good evidence to support this second assumption, as the NE in question could be made in the kidneys themselves. NE in the urine may reflect adrenergic activity not of the whole adrenergic system but of the sympathetic nerve endings in the kidney. In view of the observations recorded in this paper, further investigation of the source of urinary NE is needed.

SUMMARY AND CONCLUSIONS

The ethylenediamine method has been modified to improve its sensitivity and to make it more quantitative. The concentration in normal arterial plasma of material reacting chemically like norepinephrine (NE) is 0.72 μg per L (0.0036 μg per 5 ml), and reacting chemically like epinephrine (E) is 0.10 μg per L (0.0005 μg per 5 ml). The material called NE has an A-V difference across the extremity of − 0.08 μg per L (− 0.0004 μg per 5 ml). It has the same concentration in blood collected from the forearm and hand as in blood from the forearm alone. Its concentration in hepatic venous blood is 55 per cent of that present in arterial blood.

NE infused at a rate of 4 μg per minute has an average arterial concentration of 1.62 μg per L (0.0081 μg per 5 ml) and an average concentration in femoral venous blood of 0.46 μg per L (0.0023 μg per 5 ml). It is frequently absent in femoral venous blood. Hepatic venous blood contains only 6 per cent of that present in arterial blood.

Either the material measured in normal plasma is not NE, or NE is removed in one part of the circulation of the forearm and leg and added back to the venous blood in approximately the same concentration at a more distal point in the circulation.

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