

## Plasma Clearance of *dl*- $\beta$ -H<sup>3</sup>-Norepinephrine in Normal Human Subjects and Patients with Essential Hypertension \*

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In 1936 hemodynamic measurements in patients with essential hypertension suggested the presence of a functional increase in peripheral vasoconstriction producing an abnormally high peripheral resistance to blood flow (1). The concept that physiologic degrees of vasoconstriction were maintained by the adrenergic neurohumor, norepinephrine (NE), was proposed almost simultaneously (2), but it was nearly 10 years later that von Euler (3, 4), Holtz, Credner, and Kroneberg (5), and others demonstrated that NE was normally present in and released by mammalian sympathetic nerves. Within a year, Goldenberg and co-workers (6) suggested that essential hypertension might be caused by the simple overproduction of NE. This neurohumoral theory underwent repeated evaluation, first by the measurement of NE in urine (7, 8) and plasma (9), and later by the assay in urine of the major NE catabolites, vanillylmandelic acid (VMA) (10), normetanephrine (NM) (11), and 3-methoxy, 4-hydroxyphenyl glycol (NMG) (12).

Since no clear-cut evidence of NE overproduction could be found (8-10), and since physiologic studies suggested that essential hypertension was associated with abnormally increased vascular reactivity to infused NE (13), it seemed possible that the disease might be causally related to abnormal metabolic handling of that NE produced in normal quantities by these patients. To evaluate the metabolism of a substance that underwent degradation within moments of its administration and which produced an intense physiologic effect in nanogram amounts, a tracer compound of high

specific activity that could be given safely to human subjects was needed. In 1959, this substance, *dl*- $\beta$ -H<sup>3</sup>-NE, became available and was infused into normal volunteers and patients with essential hypertension in order to compare the rates of clearance of H<sup>3</sup>-NE and the accumulation of H<sup>3</sup>-labeled metabolites in plasma (14, 15).

### Methods

Five untreated patients fulfilling the usual criteria for the diagnosis of essential hypertension with no evidence of heart failure, azotemia, primary aldosteronism, hyperadrenocorticism, unilateral renal vascular disease, aortic coarctation, or pheochromocytoma were infused for 1 hour with 0.05  $\mu$ g *dl*- $\beta$ -H<sup>3</sup>-NE per kg of body weight per minute. Five normal subjects of comparable age and sex served as a control group. This NE dosage proved to be nonpressor in both groups. The *dl*- $\beta$ -H<sup>3</sup>-NE was derived from catalytic reduction of noradrenalone with tritium, followed by water dilution to rid the material of all exchangeable H<sup>3</sup>. Simultaneous radioassay, bioassay, and fluorimetric determinations demonstrated that the labeled compound was racemic and possessed a specific activity of up to 25 mc per mg. Paper chromatography in multiple solvent systems revealed a single symmetrical peak possessing all of the radioactivity and the same R<sub>f</sub> as standard NE. Before use, H<sup>3</sup>-NE was adsorbed on alumina, washed with water, eluted with dilute acetic acid, Seitz filtered, diluted with sterile 5% glucose in water, assayed for NE and H<sup>3</sup> content, and chromatographed in butanol:acetic acid:water (8:2:2) to demonstrate radiopurity.

Blood samples were drawn at the end of the infusion and at varying intervals during the following 24 hours. After each sample was mixed with heparin, stored for 10 minutes at 0° C, and centrifuged at 2,100 rpm for 15 minutes, the plasma was withdrawn, adsorbed on alumina, washed with ice water, and eluted with acetic acid (14). Chromatography of this eluate in two solvent systems revealed a single radioactive peak with the same R<sub>f</sub> as NE. That H<sup>3</sup> of the acid eluate could not be extracted with ethyl acetate served as further evidence of the absence of such known catabolites of NE as 3,4-dihydroxymandelic acid. Since recovery of H<sup>3</sup>-NE added to whole blood exceeded 95%, no correction factor was used. The

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sum of the eluate and effluent radioactivities in each sample was equal to the activity of the plasma. The protein of the alumina effluent was precipitated with perchloric acid. After the addition of  $K_2CO_3$  and further centrifugation, a protein-free supernatant fluid adequate for paper chromatography was obtained. Chromatography in isopropanol: $NH_3$ :water (40:9:1), butanol:acetic acid:water (8:2:2), and benzene:propionic acid:water (20:14:1) demonstrated two major radioactive peaks with  $R_f$  values comparable to those of NM and VMA. Ninety-eight per cent of labeled NM or VMA added to whole blood was recovered in the alumina effluent. A sample of the effluent was then saturated with NaCl, brought to a pH of less than 1.0 with HCl, and shaken vigorously for half an hour with 3 vol of ethyl acetate. The latter was found to contain 75% of any  $H^3$ -VMA added to whole blood, and paper chromatography of this extract in isopropanol: $NH_3$ :water and benzene:propionic acid:water revealed a single radioactive peak with an  $R_f$  the same as that of VMA.  $H^3$ -VMA assays were corrected for this recovery. No attempt was made to measure specifically  $H^3$ -NM, although it was recognized that this material accounted for most of the radioactivity remaining in the effluent after extraction of the  $H^3$ -VMA with ethyl acetate. The eluate, effluent, and ethyl acetate extract of the effluent were assayed in a dioxane-naphthalene-PPO (2,5-diphenyloxazole)-POPOP [1,4-bis-2-(5-phenyloxazolyl)benzene] phosphor in a liquid scintillation counter. Counting efficiency was determined in each sample by an internal standard and varied from 23 to 28%.  $H^3$  activity had to exceed 500 dpm per ml of plasma for reliable counting.

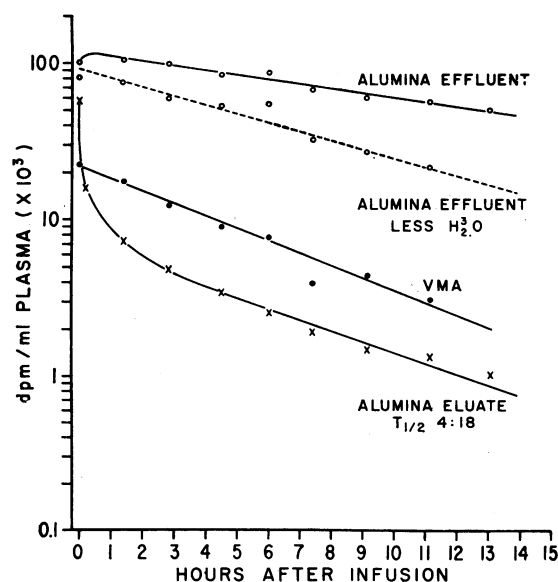


FIG. 1. PLASMA  $H^3$  CONCENTRATIONS IN ONE NORMAL SUBJECT AFTER INFUSION OF *dl*- $\beta$ - $H^3$ -NOREPINEPHRINE ( $0.05 \mu g$  per kg per minute for 1 hour). VMA = vanillylmandelic acid.

All counting was long enough in duration to reduce random counting error to less than 1%. All specimens were assayed in duplicate. Reproducibility of alumina eluate, effluent, and ethyl acetate extract activities in blood samples drawn simultaneously from different veins was always within 5%.

## Results

*Normal subjects.* The blood pressure was not increased in any patient during administration of the catecholamine. Peak plasma  $H^3$ -NE concentration was reached immediately before conclusion of the infusion, after which it fell precipitously during the first 10 minutes to approximately one-third of this value. Thereafter, the  $H^3$ -NE concentration diminished more gradually until some 3 hours later, at which time the decline became exponential, that is, rectilinear on a semilogarithmic scale (Figure 1). For each subject, the concentrations of plasma  $H^3$ -NE following the third hour after stopping the infusion were transformed into common logarithms. Correlations made between the time elapsed and the log of the  $H^3$ -NE radioactivity indicated a definite linear correspondence (Table I). Best-fitting lines were computed from the transformed data using the method of least squares (Table II). This portion of the plasma  $H^3$ -NE disappearance curve could be expressed as  $y = -0.073x + 3.85$  for the normal subjects. More simply, the average plasma  $H^3$ -NE half-time of disappearance ( $t_{1/2}$ ) was 240 minutes. Near background levels of  $H^3$ -NE were reached in about 24 to 30 hours.

Effluent  $H^3$  activity exceeded that of the eluate before the end of the infusion, and fell only slightly during the following 3 days (Figure 1). The reason for this unexpected circumstance became apparent upon distilling the samples. Despite the absence of any significant  $H^3_2O$  in the infusion, a small amount of distillable  $H^3$  appeared in the plasma before the end of the  $H^3$ -NE in-

TABLE I  
Correlation coefficients

	Normal	Essential hypertension
	0.994	0.995
	0.996	0.977
	0.968	0.979
	0.964	0.992
	0.982	0.991

jection. This remained at fairly constant levels during the ensuing hours (Table III). Evidence that the distillable H<sup>3</sup> represented H<sub>2</sub>O was obtained by conducting the distillation at 100° C, in partial vacuum at 70° C, and at - 30° C by lyophilization. These resulted in distillate of the same specific activity. Similarly, fractional collections during the first 15 minutes of distillation, redistillation of any distillate, and distillation from acidic or basic samples all resulted in distillates of constant specific activity. No H<sup>3</sup> could be extracted by hexane from the neutral distillates, and ethyl acetate extraction of acidic and basic distillates failed to remove 93.8 to 95.0% of the H<sup>3</sup> from the aqueous phase. No adsorption of H<sup>3</sup> on Amberlite CG-50 could be demonstrated. That the H<sub>2</sub>O content of the plasma remained fairly constant during the period that the eluate and effluent radioactivities diminished markedly (Table III and Figure 1) made it unlikely that the H<sub>2</sub>O represented an *in vitro* breakdown product from one of the major H<sup>3</sup>-NE catabolites. Moreover, H<sub>2</sub>O could also be demonstrated by distillation of a different biological material; an average of 5.9% of the administered H<sup>3</sup> was excreted as H<sub>2</sub>O in the urine during the 4 days following the H<sup>3</sup>-NE infusion. When the plasma effluent H<sup>3</sup> levels were corrected for their H<sup>3</sup>-VMA and H<sub>2</sub>O content, the remaining radioactivity, predominantly that of H<sup>3</sup>-NM, declined in an exponential manner approximately parallel to the last portion of the H<sup>3</sup>-NE curve. The H<sup>3</sup>-VMA concentra-

TABLE III  
*H<sub>2</sub>O in plasma after infusion of H<sup>3</sup>-norepinephrine (NE)*

Hours after end of H <sup>3</sup> -NE infusion	Noncatechol H <sup>3</sup> <i>dpm/ml plasma</i>	H <sub>2</sub> O <i>dpm/ml plasma</i>	% of H <sub>2</sub> O in effluent %
0	102,790	20,900	20.3
3	99,610	39,400	39.6
4.5	84,670	31,500	37.2
6	87,240	32,300	37.0
7.5	68,460	36,000	52.6
9	60,240	33,200	55.1
11	57,220	35,300	61.7

tions, although accounting for no more than about one-fourth of the activity of the effluent, declined in a similar fashion (Figure 1).

*Patients with essential hypertension.* Plasma H<sup>3</sup>-NE levels at the conclusion of the infusion as well as during the following 3 hours appeared similar to those of the normal subjects (Figure 2). After that time, linear correspondence could be demonstrated between the log of the H<sup>3</sup>-NE concentration and time elapsed (Table I). Best-fitting lines computed from the transformed data by the method of least squares (Table II) resulted in the mathematical expression of this portion of the plasma H<sup>3</sup>-NE disappearance curve as  $y = -$

TABLE II  
*Table of lines*

Normal subjects	
J	$y = - 0.066x + 3.94$
B	$y = - 0.068x + 3.71$
H	$y = - 0.071x + 3.98$
BR	$y = - 0.076x + 3.83$
P	$y = - 0.082x + 3.77$
Average	$y = - 0.073x + 3.85$
Hypertensive subjects	
W	$y = - 0.082x + 3.74$
C	$y = - 0.088x + 3.97$
F	$y = - 0.092x + 4.05$
JO	$y = - 0.094x + 3.96$
M	$y = - 0.141x + 3.86$
Average	$y = - 0.098x + 3.92$

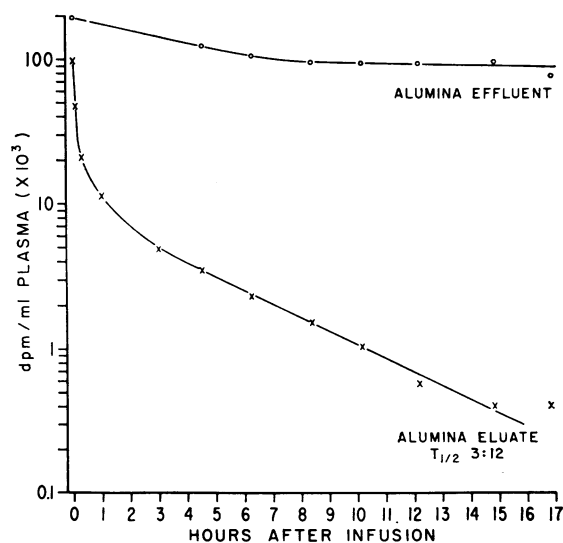


FIG. 2. PLASMA H<sup>3</sup> CONCENTRATIONS IN ONE HYPERTENSIVE SUBJECT AFTER INFUSION OF *dl*-β-H<sup>3</sup>-NOREPINEPHRINE (0.05 μg per kg per minute for 1 hour).

TABLE IV  
*Analysis of variance*

Source of variation	Degree of freedom	Sum of squares	Mean squares	F ratio	Level of significance
Between groups	1	0.001796	0.001796	5.95*	0.05
Within groups	8	0.002414	0.000302		
Total	9	0.004210			

\*  $F = 5.32$ ;  $p = .05$ .

$0.098x + 3.92$  for the hypertensive subjects. Thus the plasma  $H^3$ -NE  $t_{1/2}$  values of 215 to 258 minutes (mean, 240 minutes) for the normotensive subjects contrasted sharply with those of the hypertensive group which were only 123 to 210 minutes (mean, 183 minutes). As would be expected from this, the  $H^3$ -NE concentrations reached near-background levels in less than the normal 24 hours. The slopes of the normal and hypertensive groups were subjected to an analysis of variance to determine the degree to which their apparent difference exceeded the possibility of the results being due to random error (Table IV). It was concluded that the rate of decline of the plasma  $H^3$ -NE was significantly greater in the hypertensive than in the normal subjects (at the 0.05 level of probability). Effluent  $H^3$  levels did not differ from those noted in the control group, but the absence of  $H^3_2O$  measurements made it impossible to calculate the disappearance rates of the NE metabolites.

### Discussion

In previous studies, patients with essential hypertension were found to excrete normal quantities (8, 10, 12) of NE and its major metabolites, VMA, NM, and NMG. Moreover, no generalized defect in monamine oxidase (16) or catechol-O-methyltransferase (11, 14, 15) activity could be detected. The observations that these patients (13), as well as a relatively large number of their children (17), possessed abnormally high vascular reactivity to infused NE remained unexplained.

Although previous studies of plasma  $H^3$ -NE disappearance during the first 2 hours after infusion did not demonstrate a difference between normotensive and hypertensive subjects (15), they did reveal (14) that appreciable quantities of in-

fused NE did not undergo immediate chemical degradation but were stored in a form unavailable for immediate enzymatic degradation. Axelrod, Weil-Malherbe, and Tomchick (18) had previously detected evidence of catecholamine storage after the administration of  $H^3$ -epinephrine to mice and cats. It was therefore likely that the catecholamine-rich particulate matter found in chromaffin tissue (19, 20) and adrenergic nerves (21) constituted stores not only of locally synthesized but also previously circulating NE. Indeed, considering the facility with which the readily available enzymes could degrade NE, the persistence of measurable plasma  $H^3$ -NE hours after its injection could only indicate storage of intact NE followed by its gradual release. Other observations emphasized the importance of such storage in limiting the vasopressor action of NE. Sympathetic denervation that produces a fall in tissue NE content (22, 23) and inability of the denervated tissue to bind circulating NE (24) had long been known to be followed by hypersensitivity to injected catecholamines (25). Diminution of NE stores by reserpine and other drugs was similarly associated with increased reactivity to NE (26, 27). These observations led to the concept that neural binding of NE might serve to reduce its local concentration at the receptor sites and that a small storage pool would automatically imply deficient binding and resultant hyperreactivity to locally released or circulating NE (24). Under these circumstances, production of normal quantities of NE could conceivably result in excessive vascular reactivity.

Animal studies (28) indicated that the rapid fall in plasma  $H^3$ -NE immediately after its administration could be correlated with redistribution and conversion of the  $H^3$ -NE to its metabo-

lites, whereas the later more gradual decline in plasma  $H^3$ -NE concentration seemed dependent upon early binding of  $H^3$ -NE and its subsequent gradual release. An abnormality in the latter portion of the curve would therefore point to a defect in the binding or storage mechanism as being responsible for the observations in the hypertensive group. Similarly, many of the drugs that interfere with binding of intravenously administered  $H^3$ -NE bring about an increase in the rate of disappearance of  $H^3$ -catecholamines in the whole animal (29, 30).

Since the current techniques for determination of resting levels of plasma NE are only partially satisfactory, no effort was made to calculate plasma turnover rates. On the other hand, previous studies, however crude, have indicated that essential hypertension is associated with normal plasma NE levels (9, 15), which in conjunction with the present findings would suggest increased plasma turnover. One of the possible causes for this is a smaller than normal NE storage pool. The coexistence of a small storage pool or defective binding mechanism and increased sensitivity to infused catecholamines might then take on significance in regard to the etiology of essential hypertension. The familial nature of this disease might therefore be related to a genetically determined defect in the NE pump (31) responsible for neural storage of the catecholamine against a concentration gradient. Unfortunately, this entire circumstance is complicated by the likelihood that there is more than one pool or compartment in which NE is stored (32, 33).

The defect in  $H^3$ -NE metabolism demonstrated by these studies of patients with essential hypertension might be the result of an unusual distribution of the catecholamine due to abnormalities in circulation and therefore tissue perfusion in the hypertensive subject. Kopin, Gordon, and Horst (34) have suggested that the blood flow of any tissue determines in part its uptake of circulating labeled NE. Differences in NE turnover rates from one tissue to another might result under these circumstances in an abnormal plasma  $H^3$ -NE disappearance. Against this hypothesis is the recent observation that two patients with hypertension not of the "essential" variety failed to demonstrate an abnormality in their plasma  $H^3$ -NE disappearance (35).

Earlier studies at this laboratory indicated that plasma  $H^3$ -VMA as well as total effluent  $H^3$  activity following 30-minute infusions of  $H^3$ -NE into normal subjects and patients with essential hypertension was comparable until 3 hours after the start of the injection (15). Although the present investigation extended such observations to a 24-hour period and no differences were found between the hypertensive and control groups, the unexpected finding of  $H^3_2O$  in the effluents limited the specificity of the measurements. Sandler and Ruthven (36) have recently observed  $C^{14}$ -homovanillic acid in the urine of a subject given  $C^{14}$ -labeled NE. However, reduction of the  $\beta$  carbon, one step leading to the formation of *m*-hydroxyphenylacetic acid, was not found by Smith (37) to entail loss of the  $\beta$  tritium label. On the other hand,  $\beta$  carbon oxidation of NE was demonstrated by Rosen, Nelson, and Goodall (38) when they isolated  $C^{14}$ -labeled vanillic acid ( $C^{14}$ -VA) in the urine samples of human subjects given  $C^{14}$ -NE. Such side-chain oxidation or even complete loss of the side chain would be the likely origin of the  $H^3_2O$  observed in this study. Multiple pathways for the formation of the  $H^3_2O$  may be suspected by the fact that twice as much of this metabolite is formed as  $C^{14}$ -VA. However, this quantitative difference may be due to the tritium label.

### Summary

- 1) Five normal subjects and five patients with essential hypertension were infused with *dl*- $\beta$ - $H^3$ -norepinephrine ( $H^3$ -NE) after which the plasma  $H^3$ -NE levels were measured.
- 2) Three hours after the conclusion of the infusions the plasma  $H^3$ -NE clearances became exponential at rates uniformly more rapid in the hypertensive than in the normotensive subjects.
- 3) There is an abnormality in the dynamics of norepinephrine metabolism in patients with essential hypertension.
- 4) Tritium-labeled water is a normal catabolite of  $H^3$ -NE.

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