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THE SUBCELLULAR LOCALIZATION OF BRAIN-STEM NOREPINEPHRINE AND 5-HYDROXYTRYPTAMINE IN STRESSED RATS*

ROBERTO LEVI† and E. W. MAYNERT

Department of Pharmacology and Experimental Therapeutics,
The Johns Hopkins University School of Medicine, Baltimore, Md., U.S.A.

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Abstract—In agreement with earlier work, the total amount of brain-stem norepinephrine (NE) was subnormal in electrically shocked rats, normal in both shocked and unshocked rats sedated by phenobarbital, and supranormal in both shocked and unshocked rats treated with 1-phenyl-2-hydrazinopropane (Catron). The total amount of 5-hydroxytryptamine (5-HT) was altered only in animals receiving Catron.

Differential and gradient-density centrifugation of homogenates of rat brain stem revealed a similar localization of NE and 5-HT. About 25% of the amines were found in Whittaker's B fraction, which is composed almost entirely of nerve-ending particles.

The subcellular distribution of brain-stem NE and 5-HT was unchanged in rats treated with shock alone, with phenobarbital or Catron alone, or with shock plus phenobarbital or Catron. Exogenous NE added to a homogenate at 0° appeared entirely in the supernatant fluid.

The results of the subcellular localization experiments are interpreted in terms of the tearing of nerves during homogenization and the subsequent formation of nerve-ending particles. It is suggested that in animals treated with sedatives or monoamine oxidase inhibitors the brain-stem NE, ordinarily released by stress, fails to leave the nerve endings.

MAYNERT AND LEVI[1] reported that the large decreases in brain-stem norepinephrine (NE) which occur in rats shocked electrically fail to take place after treatment with sedative doses of chlorpromazine or phenobarbital or any of various kinds of monoamine oxidase inhibitors. Lack of information about the mechanisms of the stress induced disappearance of brain NE and the interference with this process by drugs invited a comparison of the subcellular distribution of the amine after stress, after drugs, and after the combination of stress and drugs. The hypotheses underlying this approach to the problem were as follows. First, if stress causes the release of NE from nerve endings, the loss of the amine should be reflected primarily in the nerve-ending fraction of a homogenate. Second, if pheonobarbital and chlorpromazine block nervous connections proximal to the adrenergic system of the brain stem, the nerve-ending fraction from shocked rats receiving either of these drugs should contain a normal concentration of NE. Third, if monoamine oxidase inhibitors prevent the

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† Present address: Department of Pharmacology, University of Pisa, Italy.

disappearance of NE merely by inhibiting the destruction of free amine, the nerve-ending fraction from shocked rats given a drug of this kind should have a relatively low concentration of the amine, whereas the free NE in the homogenate should be increased.

Recent work by Whittaker and his colleagues[2–4] has shown that a large part of the acetylcholine and 5-hydroxytryptamine (5-HT) in brain homogenates can be isolated in the form of nerve-ending particles which sediment at about the same rate as mitochondria in isotonic sucrose and localize between 0·8 and 1·2 M sucrose in a gradient-density tube. It seemed likely that brain-stem NE would have roughly the same properties as acetylcholine and 5-HT during differential and gradient-density centrifugation. To permit comparisons with Whittaker's data, 5-HT was measured along with NE throughout the present study.

METHODS

The rats were male Sprague-Dawley descendants weighing between 180 and 250 g. The apparatus for administering electric shocks by means of a grid floor is described elsewhere.[1] The parameters of the shock treatment and details of drug administration are given in appropriate tables. Animals were decapitated immediately after receiving the last shock, and brain stems were removed as described previously.[5]

Homogenates of four brain stems (ca. 1·8 g) in ice-cold 0·32 M sucrose were prepared in a tightly fitting all-glass apparatus and brought to a volume of 18 ml. The centrifugal forces employed in the separation of the various fractions are specified in the tables and figure legend. The P_1 fraction was separated in a size 1 International centrifuge in the cold room (2°–4°) and washed twice with 1-ml portions of 0·32 M sucrose. The P_2 and P_3 fractions were sedimented at 0° in a Spinco model L preparative ultracentrifuge with a type 50 rotor; they were not washed. The gradient density separations were made in the Spinco ultracentrifuge with a SW39L rotor. Supernatant fluids from the differential centrifugation and particulate layers A and B from the gradient-density fractionation were removed by aspiration. Values for centrifugal force given in the text were calculated from the average radii.

The method of Maynert and Klingman[5] was used for the determination of NE; 5-HT was measured by the procedure of Mead and Finger.[6] The specificity of this method was examined by subjecting the final extract from a homogenate of normal rat brain stems to a 12-tube countercurrent distribution between *n*-butanol and 0·15 M pyrophosphate buffer, pH 9·4. The results indicated that at least 90% of the fluorescence could be attributed to 5-HT. Values of NE and 5-HT given in the text refer to the free bases.

Particulate fractions were examined in an electron microscope, both as thin sections and as negative-stained suspensions.

RESULTS

In the first experiments of this investigation homogenates of brain stems from normal rats were centrifuged to remove a nuclear and debris fraction (P_1), a crude mitochondrial fraction (P_2), and a microsomal fraction (P_3). The P_2 fraction was then separated by density-gradient centrifugation. In agreement with Gray and Whittaker,[3] electron-microscopic examination revealed that the A layer was precominantly

myelin; the B layer, nerve-ending particles; and the C layer, mitochondria. Figure 1 shows that the distribution of NE among the various fractions was very much the same as that of 5-HT. The principal difference was that more 5-HT than NE (20% vs. 6%) was located in the C layer. The nerve-ending particle fraction contained 29% of the NE and 23% of the 5-HT in the whole homogenate.

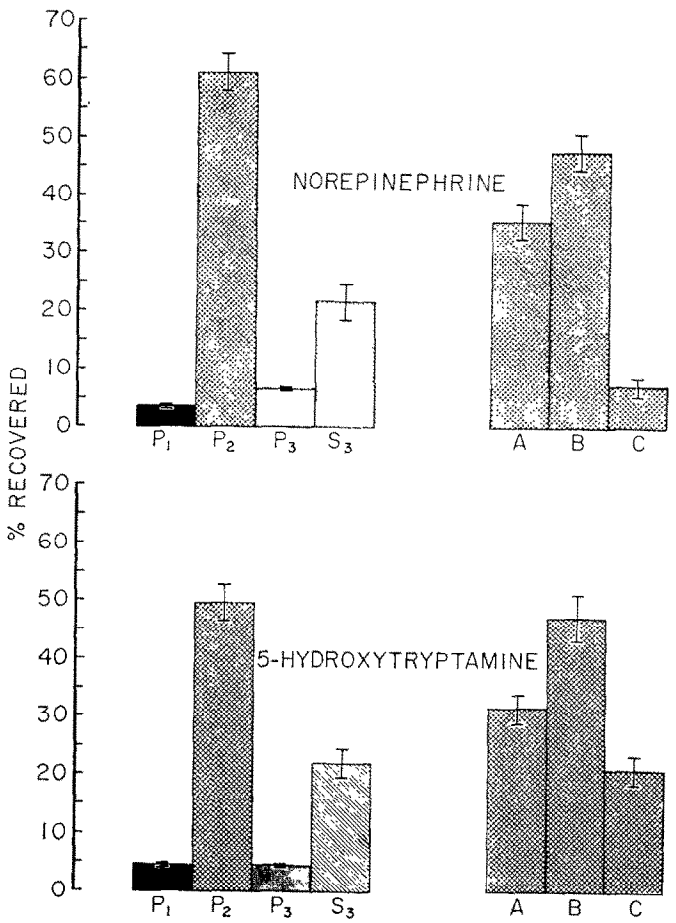

FIG. 1. The subcellular localization of NE and 5-HT in rat brain stem. P_1, P_2, and P_3 were obtained by differential centrifugation of a 0·32 M sucrose homogenate at 900 × 10, 15,000 × 30, and 110,000 × 30*g* min, respectively; S_3 = supernatant fluid from P_3. Recoveries in these fractions were calculated on the basis of analyses of the whole homogenates. The data are from 9 experiments. Fractions A, B, and C were obtained by placing a suspension of P_2 in 1 ml of 0·32 M sucrose on a discontinuous gradient composed of 2 ml of 1·2 M and 2 ml of 0·8 M sucrose, and centrifuging at 100,000 *g* for 60 min. Fraction A accumulated at the upper interface; B, at the lower; C, at the bottom of the tube. Recoveries were calculated on the basis of analyses of the P_2 suspensions. The data are from 5 experiments. The vertical bracketed lines depict ± 1 S.E.

TABLE 1. THE SUBCELLULAR LOCALIZATION OF NOREPINEPHRINE IN HOMOGENATES OF BRAIN STEM OF RAT

Treatment*	Dose (mg/kg)	No. of expts.	NE in homog. ± S.E. (μg/g tissue)	% NE recovered ± S.E.†				Total
				P_1	P_2	P_3	S_3	
Controls		12	0·67 ± 0·03	3·6 ± 0·3	61·2 ± 2·8	6·7 ± 0·1	21·5 ± 2·4	93
Shock		3	0·46 ± 0·01	4·7 ± 0·5	59·5 ± 6·9	9·3 ± 1·1	19·5 ± 5·6	93
Phenobarbital Na	100	3	0·74 ± 0·05	4·4 ± 0·4	59·5 ± 0·3	7·2 ± 0·2	23·4 ± 0·3	94
Phenobarb. + shock	100	3	0·74 ± 0·05	4·3 ± 0·4	59·9 ± 8·1	6·5 ± 0·4	22·4 ± 8·1	93
Catron	8	4	1·37 ± 0·15	4·1 ± 1·4	58·2 ± 4·9	7·2 ± 0·6	23·8 ± 6·5	93
Catron + shock	8	3	1·32 ± 0·15	4·2 ± 1·7	56·9 ± 2·1	7·4 ± 0·8	21·7 ± 0·2	90

* Catron and phenobarbital were given intraperitoneally 9 hr and 30 min, respectively, prior to administration of the shocks. The parameters of the shock treatment were: single shock duration, 1 sec; shock frequency, 6 per minute; treatment time, 1 hr. Shocked and unshocked rats were killed at the same time after drug administration.

† P_1, P_2, and P_3 were obtained by differential centrifugation of a 0·32 M sucrose homogenate at 900 × 10, 15,000 × 30, and 110,000 × 30 *g* min, respectively; S_3 = supernatant fluid from P_3. Recoveries in these fractions were calculated on the basis of analyses of the whole homogenates.

TABLE 2. THE SUBCELLULAR LOCALIZATION OF 5-HYDROXYTRYPTAMINE IN HOMOGENATES OF BRAIN STEM OF RAT

Treatment*	Dose (mg/kg)	No. of expts.	5-HT in homog. ± S.E. (μg/g tissue)	% 5-HT recovered ± S.E.*				Total
				P_1	P_2	P_3	S_3	
Controls		12	0·56 ± 0·05	4·5 ± 0·4	49·4 ± 3·3	4·7 ± 0·2	22·0 ± 2·8	
Shock		3	0·55 ± 0·02	7·2 ± 0·3	49·6 ± 1.9	6·0 ± 0·1	28·2 ± 1.9	91
Phenobarbital Na	100	3	0·52 ± 0.05	6·6 ± 0.3	51·1 ± 6.9	6·2 ± 1.0	24·8 ± 0·4	89
Phenobarb. + shock	100	3	0·53 ± 0.04	6·1 ± 0·5	54·1 ± 4.9	6·1 ± 1·0	25·7 ± 1·9	92
Catron	8	4	1·62 ± 0·33	5·6 ± 0·4	52·3 ± 3·0	6·0 ± 0·6	26·4 ± 1·2	90
Catron + shock	8	3	1·69 ± 0·03	5·0 ± 0·1	49·8 ± 7·1	6·1 ± 1·9	25·6 ± 1·9	87

* See footnotes to Table 1.

A comparison of the data in Fig. 1 with results obtained by others is made somewhat tenuous by the variety of species, brain parts, conditions for the separation of fractions, and analytical methods employed. Nevertheless, it may be noted that the distribution of 5-HT after differential centrifugation is in harmony with most reports, [2, 4, 7–9] although it differs from those of Zieher and De Robertis,[10] Ryall,[11] and (to some extent) Carlini and Green.[12] These three groups of workers found rather large amounts of 5-HT in the P_3 fraction. Studies of the differential centrifugation of NE[13–16] have generally involved the separation of only one or two particulate fractions. In this older work usually more (up to 46%) of the amine was found in a soluble form. The present data from density-gradient centrifugation reveal slightly more of the 5-HT in the A and C layers than was found by most workers[2, 4, 10–12] but decidedly less than was found by Kataoka.[9] The literature on gradient-density fractionation of endogenous NE consists of two preliminary communications [17, 18] without quantitative data and a recent note[19] indicating a parallelism between NE and 5-HT.

The investigation of the subcellular localization of NE and 5-HT in shocked and drug-treated rats was confined to the fractions derived from differential centrifugation. The results in Tables 1 and 2 reveal that the distribution of the amines was not changed by shock alone, by phenobarbital or Catron alone, or by shock plus phenobarbital or Catron. The data on total NE and 5-HT agree with earlier work.[1] Thus the shock treatment caused a 31% decrease in NE but no change in 5-HT. Phenobarbital alone had no obvious effect on either NE or 5-HT, but it was completely effective in preventing the shock-induced decrease in NE. In unshocked animals Catron markedly increased both NE and 5-HT; it also inhibited the shock-induced decrease in NE.

The results in Table 1 are in agreement with the observation of Weil-Malherbe *et al.*[20] that the ratio between particle-bound and soluble NE in homogenates of rabbit brain was not changed by Catron. However, the data in Table 2 are not in accord with Schanberg and Giarman's findings[21] with the clam heart assay that phenobarbital caused an increase in total 5-HT with a greater increase in free than in bound amine. These authors also found that Catron caused a borderline decrease in the ratio of free to bound 5-HT.

The discovery that the distribution of NE and 5-HT was not changed by any of the treatments employed in this study suggested an equilibrium between particle-bound and 'free' amines. This possibility was examined by adding NE to a cold (0°) brain-stem homogenate, which was then immediately subjected to differential centrifugation. The data in Table 3 show that the exogenous NE was recovered entirely in the supernatant fluid. These results are in harmony with the observation[15] that particulate fractions can be washed repeatedly in the cold without a detectable loss of NE.

DISCUSSION

Several investigators[14, 15, 20, 21] have attempted to interpret the actions of drugs in terms of their effects on the subcellular localization of brain NE and 5-HT. However, almost no attention has been given to the probable nature of either the particle-bound or the 'free' amines. In these studies over 25% of the NE and 5-HT has been found to be 'free'. Although proof is lacking, it appears unlikely that so large a fraction of these amines occurs extracellularly in the brain. In view of the large blood supply and the presence in brain of enzymes capable of destroying NE and 5-HT, extracellular

amines would be expected to disappear quickly. Maynert and Levi[1] showed that electric shocks applied to the feet of rats caused a 17% decrease in brain NE in 5 min. The easiest explanation for this observation is that stress causes the release of NE from nerve endings and that the released amine is rapidly eliminated.

TABLE 3. THE LOCALIZATION OF NOREPINEPHRINE ADDED TO A HOMOGENATE OF RAT BRAIN STEMS AT 0°*

Fraction	Homog. (ng NE)	Homog. + 500 ng NE (ng NE)	Difference (ng NE)
P_1	63	86	+23
P_2	912	905	−7
P_3	120	110	−10
S_3	215	715	+500
$P_1 + P_2 + P_3 + S_3$†	1,310	1,816	+506

* P_1, P_2 and P_3 were obtained by differential centrifugation at 900 × 10, 15,000 × 30, and 110,000 × 30 *g* min, respectively. S_3 = supernatant fluid from P_3. The centrifugation was begun immediately after the addition of the exogenous NE to the homogenate. The data are averages from two experiments.

† By calculation.

The discovery of the nerve-ending particle has not only provided evidence that substances like acetylcholine, 5-HT, and NE occur largely in nerves but appears to offer a simple explanation for the 'free' amines. During homogenization the nerve endings are torn from the remainder of the nerve, and before they become sealed[3] part of their contents leaks into the surrounding fluid. It might be expected that the formation of nerve-ending particles would be determined largely by anatomical factors (and perhaps to some extent by the rigorousness of homogenization) and that the same fraction of the neuronal contents would escape regardless of whether the amine content was subnormal or supranormal. The same situation would obtain whether all the intraneuronal amine was bound within synaptic vesicles or whether there was an equilibrium between unbound and vesicular-bound amine. However, if all the amine were bound in vesicles, it would be necessary to postulate that the released vesicles are lysed in isotonic sucrose. Recent experiments* on isolated vesicles[22, 23] have shown that such lysis is not extensive. Therefore it seems probable that within nerve endings substances like acetylcholine, 5-HT, and NE are partly free and partly bound and that an equilibrium obtains between the free and the bound forms.

The results of the present investigation fit this framework. Despite marked changes in the total concentration of the amines, the subcellular distribution of NE and 5-HT was not altered by shock, or phenobarbital, or Catron, or by the combination of either drug and shock. That the data could not be explained by equilibria between the particulate fractions and the soluble amines was proved by adding NE to a homogenate at 0° and subsequently recovering all of it in the supernatant fluid. Inasmuch as the brain stems analyzed in this study were always cooled to 0° prior to homogenization,

* E. W. Maynert, R. Levi and A. J. D. Lorenzo; unpublished observations.

observations[24–26] that brain slices or particulate matter can accumulate NE or 5-HT at normal body temperatures would not appear to be directly relevant.

To a certain extent the arguments outlined above preclude answers to the questions which initiated this investigation of the subcellular distribution of NE. Nevertheless, the data are compatible with the hypotheses that electric shocks cause the release of NE from nerve endings and that drugs like phenobarbital prevent the release. The observations on Catron-treated animals are difficult to reconcile with the view that monoamine oxidase inhibitors merely prevent the destruction of released amine[27]. It appears more likely that this drug also prevents the stress-induced release of NE from nerve endings. However, the primary site of action need not be the same as that of phenobarbital. Kopin and Gordon[28] have recently reviewed other evidence suggesting that monoamine oxidase inhibitors hinder the liberation of neuronal NE.

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