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FACTORS INFLUENCING THE UPTAKE OF NORADRENALINE BY SUBCELLULAR PARTICLES IN HOMOGENATES OF RAT BRAIN*

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Abstract—A preparation of granules obtained from rat brain by differential centrifugation has been investigated for characteristics of release and uptake of catecholamines. Activation and fluorescence spectra obtained from chromatographically isolated extracts of such granules indicated that noradrenaline is the predominant endogenous catecholamine. The rate of release of this endogenous noradrenaline varied directly with temperature of incubation. When the brain granules were incubated with exogenously added noradrenaline, uptake varied directly with the external concentration of the amine, with partial saturation appearing at concentrations beyond 200 mμg noradrenaline/ml incubation medium. Partial depletion of noradrenaline from brain granules by incubation at 37° did not alter the capacity of the granules to take up exogenous noradrenaline. Greater actual uptake of exogenous noradrenaline by the granules occurred at 37° than at 4°, even though total uptake was less at 37°. Administration of reserpine to the rats markedly decreased endogenous levels of noradrenaline in the brain granules. Although granules from reserpine-treated animals continued to demonstrate an uptake of exogenous noradrenaline, the total quantity taken up was markedly below that in control preparations. Neither dinitrophenol nor iodoacetic acid altered the uptake of exogenous noradrenaline by the brain granules.

THE localization of endogenous catecholamines in subcellular particles obtained from homogenates of the central nervous system,[1, 2] the adrenal gland,[3–5] and postganglionic sympathetic nerve fibers (splenic nerves)[6–8] has been previously described. Exogenous catecholamines may also be concentrated and bound by intra-axonal particles, as has been demonstrated by incubations *in vitro* with subcellular fractions prepared from the adrenal gland of various animals,[9–11] bovine splenic nerve,[12, 13] and the brain of the rat.[14, 15] The uptake of infused catecholamine *in vivo* by neuronal structures, after pretreatment with reserpine, has been shown temporarily to restore autonomic effector reactivity to nerve stimulation.[16–18] These observations suggest that cytoplasmic granules within the neuron may fulfill an integral function in the synthesis, storage, and release of adrenergic transmitter under physiological conditions.

In the present investigation particulate fractions prepared from rat brain homogenates have been utilized to study factors influencing catecholamine release and uptake by subcellular structures.

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METHODS

Preparation of subcellular fractions

Freshly excised whole brains obtained from decapitated male rats (Sprague-Dawley, ca. 200 g) were homogenized for 65 sec at 4° in a glass homogenizer (Teflon pestle), containing an aqueous medium of sucrose (0·27 M) and edathamil (Versene, 0·1%); the final ratio of the sucrose medium to brain tissue (wet weight) was 9 : 1. All centrifugation procedures were performed at 4°. Nuclei and cell debris were removed by centrifugation at 900 *g* for 10 min. The cloudy supernatant fraction (25-ml aliquot) was decanted and incubated with the priming amine at 37° for variable periods depending upon the requirements of the particular experiment. The 900 *g* supernatant fraction has been referred to as the *brain granules* fraction in the text. It was probably composed of a nonhomogeneous mixture containing mitochondria, microsomes, and myelin particles as demonstrated by Michaelson and Whittaker.[19] Metabolic inhibitors such as iodoacetic acid (IAA) or dinitrophenol (DNP) were also added at this time in certain experiments.

Attempts to maintain the suspensions at physiological pH with phosphate buffer or Tris in 0·27 M sucrose failed because the presence of the buffer seemed to alter the characteristics of the brain granules, resulting in great variability in the uptake of the amine. The pH of the unbuffered suspensions varied by less than 0·1 unit from pH 6·8, when measurements were made at various times from the completion of the homogenate to the end of the incubations.

Extraction of catecholamine

At the end of the desired incubation period the mixture was centrifuged in a Spinco preparative ultracentrifuge at 100,000 *g* for 30 min. The high-speed supernatant fraction was decanted and the sediment drained by inversion of the tube. The walls of the tube were wiped free of all remaining supernatant fluid. In some experiments the high-speed sediment was resuspended in 0·27 M sucrose and further centrifuged; such specimens are referred to as *resuspended brain granules*. Values obtained after this treatment did not differ significantly ($P < 5\%$) from particles not resuspended.[14] The sediment was deproteinized by the serial addition of 3 aliquots of 0·4 N perchloric acid (2 ml each). The mixture was then centrifuged for 5 min at 900 *g*, producing a hard precipitate; the clear supernatant fluid was passed through a cationic-exchange column (Dowex 50-X8),[20] acetate buffered at pH 6·0, and the catecholamines eluted with 1 N HCl. Several samples were chromatographed on paper strips in a phenol–HCl solvent system.[21]

Quantitative estimation of catecholamine

The noradrenaline, and occasionally the adrenaline, content was estimated fluorometrically by a modification of the method of von Euler and Lishajko.[22] Ethylenediamine was added to the ascorbate–sodium hydroxide solution to stabilize the fluorophore. Assays were performed by means of a Turner fluorometer (model 110) with a microcuvette adaptor and the following filters: primary, Turner 110-811 (peak at 360 mμ); secondary, Turner 110-818 (sharp cut at 510 mμ). Scanning for fluorescence and activation spectra were performed with an Aminco–Bowman spectrophotofluorometer.

Drugs

All concentrations of catecholamines are given in terms of the free base. Solutions were prepared on the day of the experiment from frozen stock solutions (−20°) containing 1 mg noradrenaline (base)/ml 0·1 N HCl.

RESULTS

Identification of endogenous catecholamine in brain granules as noradrenaline

Brain granules prepared as described in Methods were analyzed for their catecholamine content prior to drug treatment and/or incubation with exogenous substrate. Fluorescence and activation spectra obtained from extracts of these fractions, after ion-exchange and paper chromatographic separation of the catecholamines, closely resembled those produced by noradrenaline. The chromatographic eluates did not appear to contain adrenaline in any significant or consistently reproducible quantities, and consequently no systematic effort was made to distinguish alterations in the levels of this catecholamine.

Heat-induced release of endogenous noradrenaline from brain granules

The rate of release of endogenous noradrenaline from incubated granules varied as a direct function of the incubation temperature. Brain granules maintained at 4° exhibited relatively small losses of particle-bound noradrenaline after 30–60 min of

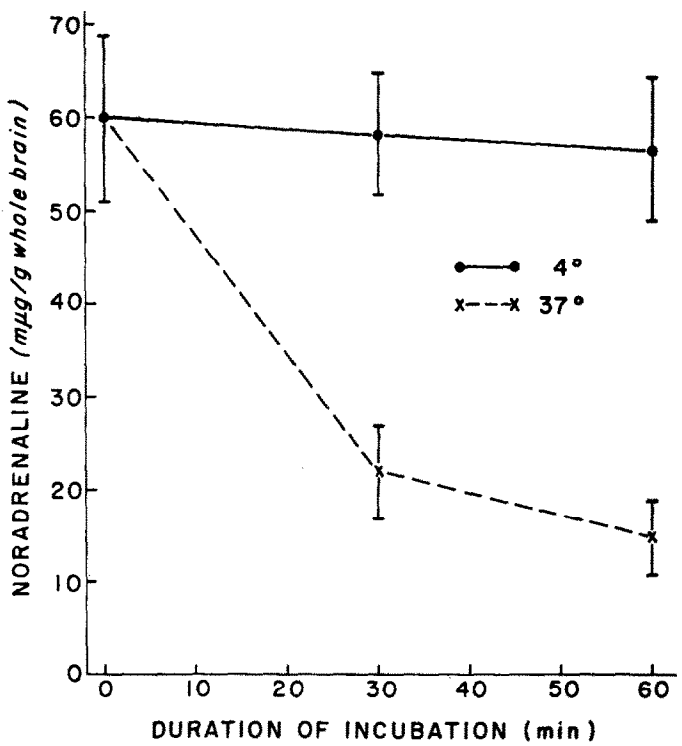

FIG. 1. Effect of incubation temperature on the spontaneous loss of particle-bound noradrenaline from brain granules. Data taken from 10 experiments. Vertical bars represent standard error of the mean.

incubation. In contrast, granules incubated at 37° were rapidly and extensively depleted of their endogenous noradrenaline content. A partial release of particle-bound noradrenaline at this temperature was observed after 10 min incubation and appeared to be near maximal at 60 min. A 30-min incubation at 37° lowered endogenous particle-bound noradrenaline levels to 63% of control (zero time of incubation) values; further incubation, up to 60 min, increased the extent of depletion to 75% of the controls (Fig. 1).

Uptake of exogenous noradrenaline by brain granules

Incubation of brain granules in media containing different concentrations of noradrenaline demonstrated that exogenous catecholamine could be taken up by such preparations. The extent of the uptake at the end of 30 min appeared to be directly proportional to the external concentration of noradrenaline. A slight increase in granule-bound noradrenaline was observed after incubation with noradreanline at levels of 20 mμg/ml. In most experiments noradrenaline at a concentration of 40 mμg/ml was used as the threshold priming dose, and significant uptake was observed in every instance. A marked decrease in the slope of the uptake curve occurred at noradrenaline concentrations beyond 200 mμg/ml, suggesting some partial saturation of the binding (uptake) mechanism (Fig. 2).

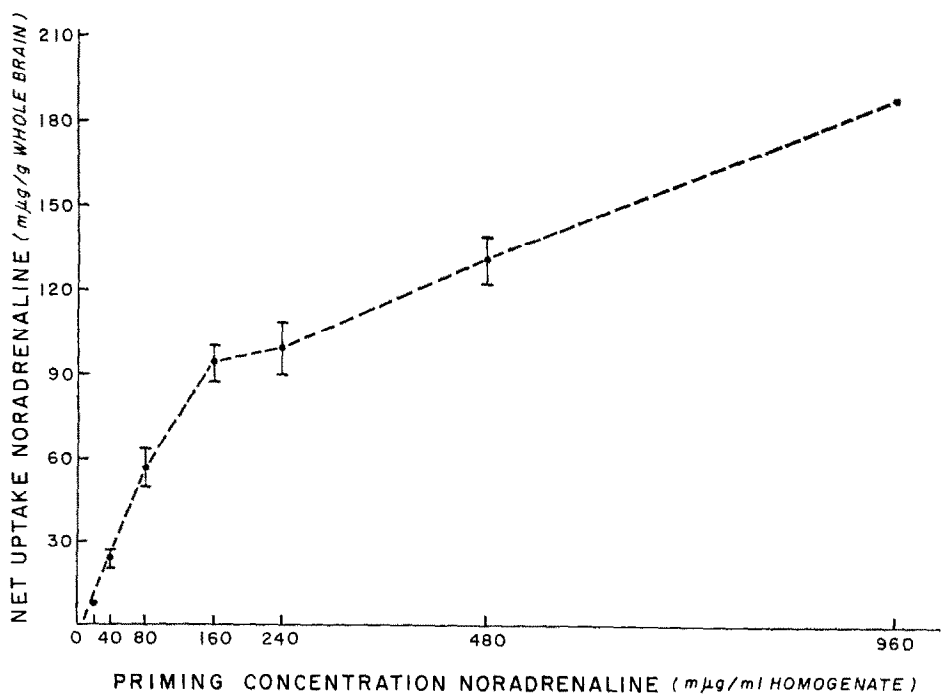

FIG. 2. Effect of exogenous noradrenaline concentration on particle-bound noradrenaline in brain granules after incubation at 37° for 30 min. Vertical bars represent standard error of the mean.

The rapid spontaneous loss of endogenous particle-bound noradrenaline occurring at 37° was prevented by the addition of adequate quantities of noradrenaline to the incubation media. Thus, incubation of brain granules with noradrenaline (80 mμg/ml) for 30 min at 37° maintained granule-bound noradrenaline slightly above control

(zero time of incubation) levels. These results accord with those of von Euler and Lishajko,[13] who prevented spontaneous loss of noradrenaline from resuspended splenic nerve granules by the addition of noradrenaline (100–300 mμg/ml) to the incubation media.

Repletion studies were carried out on untreated granule preparations incubated for 30 min at either 4° or 37°, after which noradrenaline (40 mμg/ml) was added and an additional 30-min incubation carried out. These investigations demonstrated a marked uptake of noradrenaline in partially depleted granules, occasionally to levels above control values, and also indicated that such preparations could be suspended in sucrose media for periods from 60 to 120 min without appreciably altering their capacity for amine uptake (Fig. 3).

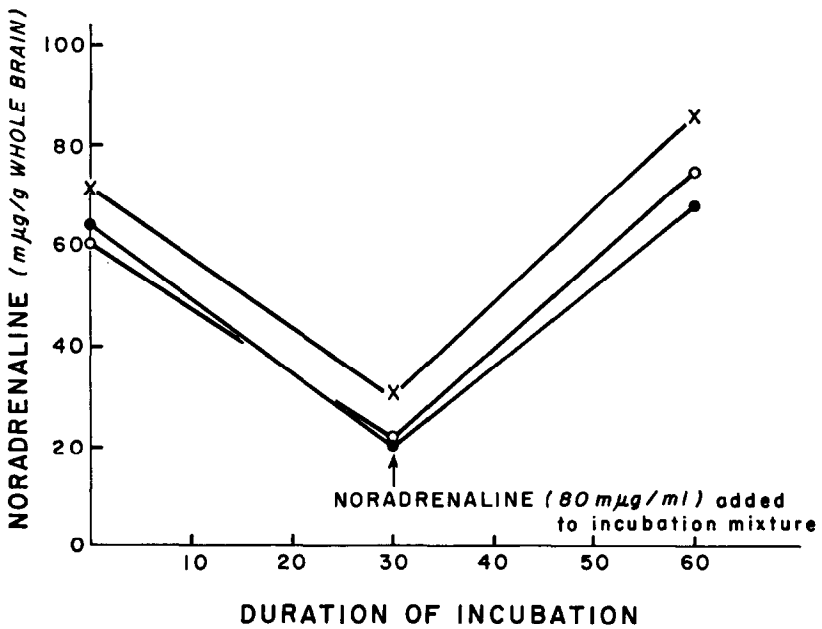

FIG. 3. Repleting effect of exogenous noradrenaline on particle-bound noradrenaline in previously depleted brain granules. Fractions were incubated at 37° for 30 min in media containing no exogenous amine. Noradrenaline (80 mμg/ml) was added at 30 min and incubation carried out for an additional 30 min at 37°. Data taken from three different repletion experiments.

Effect of temperature on uptake of noradrenaline by brain granules

Aliquots of a suspension of brain granules were incubated at either 4° or 37° in the presence of exogenous noradrenaline (40 mμg/ml). These experiments demonstrated that the *total* noradrenaline content of granules incubated at 4° for 30 or 60 min was consistently greater than that of granules suspended at 37°. Estimation of the *actual uptake** of exogenous noradrenaline revealed that, although total noradrenaline levels were much lower at 37° as compared to 4°, a significantly greater quantity of noradrenaline was taken up at 37° during the initial 30-min incubation. Thus, after

* Actual uptake = brain granules after incubation with noradrenaline-enriched media − particle-bound noradrenaline in brain granules after incubation in media devoid of priming amine.

30 min of incubation the actual uptake of noradrenaline was estimated as 24 mμg/g whole brain (range, 22–27) at 37°, contrasted to 11·5 mμg/g whole brain (range, 6–17) at 4°; total noradrenaline content at this time was 34 mμg/g whole brain (range, 29–42) at 37° and 100 mμg/g whole brain (range, 96–105) at 4°. This pattern was reversed after a 60-min incubation, so that the *actual uptake* at 4° of 33 mμg noradrenaline/g whole brain (range, 15–51), was greater than the 18 mμg noradrenaline/g whole brain (range, 0–21) obtained at 37°. These results at 60 min may be misleading, since they suggest a greater actual rate of uptake at 4° than at 37°, whereas in all probability the enhanced rate of release at 37° is the main determinant for this difference.

Effect of reserpine, DNP, and IAA on uptake of noradrenaline by brain granules

The administration of reserpine (5 mg/kg i.p.) 24 hr before decapitation markedly decreased the endogenous noradrenaline content of brain granules. In three such preparations noradrenaline concentrations before incubation ranged from 9 to 21 mμg/g whole brain compared to control values of 60 mμg/g whole brain. Incubation of granules, prepared from reserpine-treated animals at 37° for 30 min caused almost 100% release of endogenous noradrenaline within this period. The addition of priming amine (noradrenaline, 40 mμg/ml) to the incubation media at zero time or after 30 min preincubation resulted in a marked uptake of exogenous amine (Fig. 4). In all

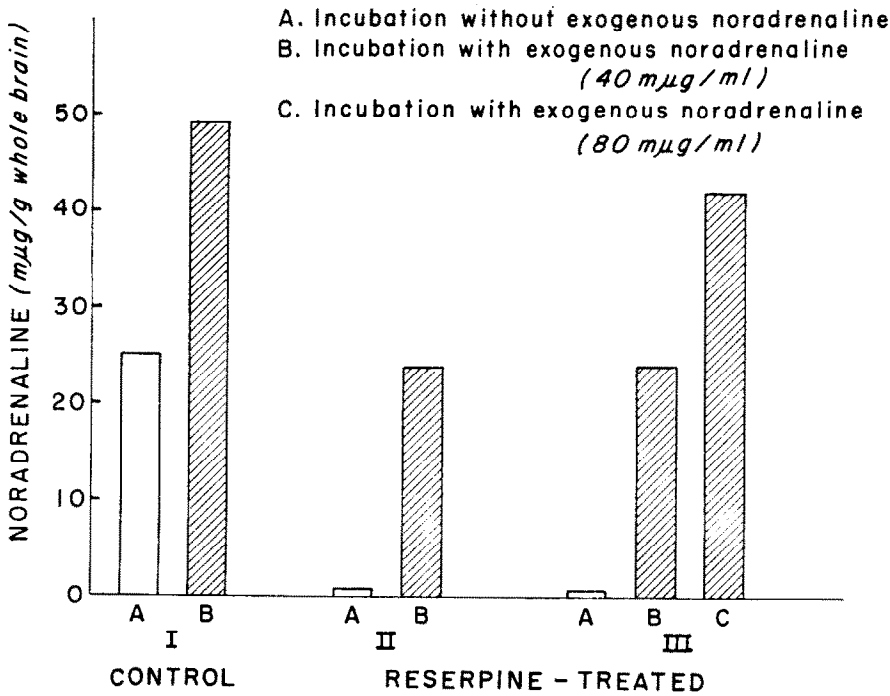

FIG. 4. Effect of reserpine (5 mg/kg i.p. 24 hr before decapitation) on particle-bound noradrenaline in brain granules incubated without (clear) and with (hatched) exogenous noradrenaline. Incubation time 30 min; temperature 37°. Experiments involving control (I) and reserpine-treated (II and III) aniamsl were performed simultaneously. Exogenous noradrenaline concentrations as follows: A, no priming amine added; B, noradrenaline (40 mμg/ml) added; C, noradrenaline (80 mμg/ml) added.

experiments, however, the total quantity of exogenous noradrenaline incorporated as particle-bound material was markedly below that in control (nonreserpinized) preparations.

The addition of DNP (1×10^{-4} M) or IAA (1×10^{-3} M) to the incubation media did not inhibit the uptake of noradrenaline by brain granules. In three experiments with these compounds it was noted that the uptake of noradrenaline was slightly greater than in control incubation mixtures.

DISCUSSION

Activation and fluorescence spectra obtained from chromatographically isolated extracts of brain granules indicate that noradrenaline is the predominant endogenous catecholamine, with very small quantities of adrenaline also present. The average content of noradrenaline in these granule fractions was 60 ± 8 mμg/g whole brain. A similar predominance of noradrenaline relative to adrenaline has been previously demonstrated in subcellular particles obtained from adrenergically innervated structures such as spleen,[6] heart,[23] and brain.[1, 2] It is probable that dopamine is also present in these granules; however, no attempt was made to analyze for this substance in the present investigation.

The spontaneous rate of release of endogenous noradrenaline varied directly as a function of the incubation temperature. Spontaneous release was markedly retarded at 4° (5% release after 60 min incubation), whereas suspension of brain granules at 37° resulted in severe depletion (75% release after 60 min incubation). The poor retention of endogenous catecholamine by rat brain granules at 37° (also by cat adrenal medullary granules[24]) closely parallels that reported for bovine splenic nerve granules[12] and is in sharp contrast to the high retention observed in rabbit and bovine adrenal medullary granules incubated at 37°.[4, 25] Curiously, the release of catecholamines from *tissue slices* of the fowl's adrenal gland appears to have a temperature–release curve similar to that of isolated brain and splenic nerve granules.[26] It appears that in some species a greater degree of cellular integrity and organization is required for the spontaneous release of noradrenaline from adrenal medullary granules at 37° than is necessary for comparable release from brain or splenic nerve granules. In this regard it is noteworthy that acetylcholine is ineffective in releasing catecholamines from splenic nerve or adrenal medullary granules[27] but is able to do so in adrenal medullary tissue slices obtained from the guinea pig.[28]

A survey of noradrenaline-uptake curves obtained after incubation of brain granules with exogenous noradrenaline suggests that the 'normal' catecholamine saturation of binding sites in these subcellular particles is incomplete. Since it is probable that these granules represent a main storage site for intracellular catecholamines, the relatively low 'normal' saturation of such organelles would seem to be consistent with a rapid synthesis and exchange of catecholamines between particle-bound and extra granular pools, thereby providing adequate quantities of transmitter substances for discharge upon neuronal excitation. Some additional evidence has been obtained in studies with more physicologically integrated model systems—i.e. postganglionic adrenergic neuroeffector units—which also favors the foregoing conception. Thus, relatively constant levels of transmitter substance are obtained in venous effluents[21] and in tissue extracts[29, 30] of adrenergically innervated organs after prolonged periods

of sympathetic nerve stimulation. The rapid (within 20 sec) though temporary restoration of adrenergic neuron reactivity to electrical stimulation in reserpine-treated cats, induced by small quantities of noradrenaline (or precursors), implies immediate assimilation of this exogenous material into a state from which it may be released at the nerve terminal.[16]

The uptake and release of noradrenaline by brain granules was not influenced by DNP (1×10^{-4} M) or IAA (1×10^{-3} M). These results agree with those of other investigators who have shown that DNP exerts no effect upon catecholamine uptake in adrenal medullary granules,[31] catecholamine release in adrenal medullary slices,[26] and 5-hydroxytryptamine uptake in rabbit brain granules.[32] The uptake of 5-hydroxytryptophan by rat brain *tissue slices* was inhibited by DNP,[33] emphasizing again the effect of cellular organization on uptake phenomena. A similar dichotomy of drug effect apparently influenced by the state of cellular disruption is seen in the action of ouabain, which diminished the uptake of ^{3}H-noradrenaline in pineal *tissue slices*[34] but had no effect on ^{3}H-noradrenaline uptake by the pineal body *in vivo*.[35]

Reserpine-treated animals had significantly depressed levels of endogenous noradrenaline in granule preparations made from their brains, but these granules were able to take up exogenous noradrenaline. Further analysis of such granule preparations by means of ^{3}H-noradrenaline and density-gradient separation has shown that reserpine decreases not only total uptake capacity but selective distribution of ^{3}H-noradrenaline into the microsomal fraction as well.[15]

At present, the role of energy-dependent active transport mechanisms in catecholamine uptake by isolated granule systems seems unsettled, owing to the conflicting nature of available evidence. Thus, uptake of catecholamines by brain granules, splenic nerve granules,[6-8] and adrenal medullary granules[10, 36] is somewhat nonspecific and quite refractory to inhibitors of oxidative phosphorylation; yet, uptake in adrenal medullary granules can be stimulated by ATP and magnesium,[37] whereas it is inhibited in *tissue slices* of cat cerebral cortex,[38] brain granules,[14, 15] and adrenal medullary granules[37] by treatment with reserpine or incubation at 4°. From these observations it is apparent that subcellular particles containing catecholamines vary greatly in their overall reactivity to environmental influences. This variation probably reflects fundamental differences in their manner of binding catecholamines and their metabolic composition.

REFERENCES

1. A. BERTLER, N. A. HILLARP and E. ROSENGREN, *Acta physiol. scand.* **50**, 113 (1960).
2. H. WEIL-MALHERBE and A. D. BONE, *J. Neurochem.* **4**, 251 (1959).
3. H. BLASHKO and A. D. WELCH, *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **129**, 17 (1953).
4. N.-A. HILLARP and B. NILSON, *Acta physiol. scand.* **31**, Supp. 113 (1954).
5. N.-A. HILLARP, *Acta physiol. scand.* **47**, 271 (1958).
6. U. S. VON EULER and N.-A. HILLARP, *Nature, Lond.* **177**, 44 (1956).
7. H. SCHUMANN, *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **234**, 17 (1958).
8. U. S. VON EULER, *Acta physiol. scand.* **43**, 155 (1958).
9. A. BERTLER, G. HALL, N.-A. HILLARP and E. ROSENGREN, *Acta physiol. scand.* **52**, 167 (1961).
10. A. BERTLER, A. ROSENGREN and E. ROSENGREN, *Experientia (Basel)* **16**, 418 (1960).
11. A. CARLSSON and N.-A. HILLARP, *Med. exp. (Basel)* **5**, 122 (1961).
12. U. S. VON EULER and F. LISHAJKO, *Acta physiol. scand.* **51**, 193 (1961).
13. U. S. VON EULER and F. LISHAJKO, *Acta physiol. scand.* **57**, 468 (1963).
14. B. L. MIRKIN, N. J. GIARMAN and D. X. FREEDMAN, *Biochem. Pharmacol.* **12**, 214 (1963).

15. B. L. MIRKIN and C. N. GILLIS, *Biochem. Pharmacol.* **12**, 1173 (1963).
16. B. L. MIRKIN and U. S. VON EULER, *J. Physiol. (Lond.)* **168**, 804 (1963).
17. S. ROSELL and G. SEDVALL, *Acta physiol. scand.* **53**, 174 (1961).
18. J. H. BURN and M. J. RAND, *J. Physiol. (Lond.)* **144**, 314 (1958).
19. I. A. MICHAELSON and U. P. WHITTAKER, *Biochem. Pharmacol.* **11**, 505 (1962).
20. A. BERTLER, A. CARLSSON and E. ROSENGREN, *Acta physiol. scand.* **44**, 273 (1958).
21. B. L. MIRKIN and D. D. BONNYCASTLE, *Amer. J. Physiol.* **178**, 529 (1954).
22. U. S. VON EULER and F. LISHAJKO, *Acta physiol. scand.* **51**, 348 (1961).
23. A. WEGMANN and K. KAKO, *Nature, Lond.* **192**, 978 (1961).
24. U. S. VON EULER and F. LISHAJKO, *Acta physiol. scand.* **52**, 37 (1961).
25. N. R. EADE, *Brit. J. Pharmacol.* **12**, 62 (1957).
26. P. R. DRASKOCZY, W. BURACK and N. WEINER, *J. Pharmacol. exp. Ther.* **141**, 58 (1963).
27. H. BLASCHKO, P. HAGEN and A. D. WELCH, *J. Physiol. (Lond)* **129**, 27 (1955).
28. M. R. HOKIN, B. G. BENFEY and E. HOKIN, *J. biol. Chem.* **233**, 814 (1958).
29. J. V. LUCO and F. GONI, *J. Neurophysiol.* **11**, 497 (1948).
30. U. S. VON EULER and S. HELLNER-BJORKMAN, *Acta physiol. scand.* **33**, Supp. 118, 17 (1955).
31. A. CARLSSON, N.-A. HILLARP and B. WALDECK, *Med. exp. (Basel)* **6**, 47 (1962).
32. A. INOUYE, K. KATAOKA and J. SHINAGAWA, *Nature, Lond.* **198**, 292 (1963).
33. S. SHANBERG and N. J. GIARMAN, *Biochim. biophys. Acta* **41**, 556 (1960).
34. H. J. DENGLER, C. WILSON, H. SPIEGEL and E. TITUS, *Biochem. Pharmacol.* **11**, 795 (1962).
35. H. WEIL-MALHERBE, L. WHITBY and J. AXELROD, *J. Neurochem.* **8**, 55 (1961).
36. H. J. SCHUMANN and A. PHILIPPU, *Nature, Lond.* **193**, 890 (1962).
37. N. KIRSCHNER, *Science* **135**, 107 (1961).
38. H. J. DENGLER, H. SPIEGEL and E. TITUS, *Science*, **133**, 1072 (1961).