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H. SUMMARY OF DISCUSSION AND COMMENTARY

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Three types of method have been presented here, all having their own particular applications, advantages, and limitations: the superfusion technique for bioassay, chemical methods based on isolation and determination by spectrophotofluorescence, and fluorescence microscopy.

As pointed out by Vane, the superfusion bioassay technique permits the immediate, continual determination of the levels of epinephrine (E), norepinephrine (NE), and other biologically active substances in blood. With respect to the waves or bursts of E secretion noted in the cat following the injection of bradykinin, Feldberg suggested that since the adrenal had not been denervated, this phenomenon might have been due to a central action; he has noted this type of response only after injections of snake venom which caused marked cellular destruction. In reply to Kirshner's question, Vane indicated that after the injection of peptides and histamine, the cat adrenal medulla releases mostly E and very little NE, in spite of its proportionately high content of the latter. Booker found that the sensitivity of the guinea pig heart to angiotensin was independent of its catecholamine content. While using this preparation to assay recovery of E or NE from dog blood during continuous infusion at a rate of 1.5 μg per kg per min, Booker noted a close similarity between the results obtained with crude plasma and those with plasma extracts, prepared by the method of von Euler and Floding, both being in agreement with the values determined fluorimetrically. Pekkarinen indicated that the distal segment of the hen's rectal cecum is more sensitive than the proximal for the assay of E; the addition of 1 mg ascorbic acid per 100 ml Tyrode solution stabilizes the E and greatly increases the sensitivity of the assay.

Although the basic methods used most commonly for the fluorimetric determination of catecholamines and their metabolites are still essentially the same as those reported at the time of the previous symposium, a vast amount of work has been done since then on their refinement, along with the introduction of some newer techniques.

The following comments were made from the floor in discussion of the papers of Häggendal, Weil-Malherbe and Smith, and Sandler and Ruthven.

BRUNJES: "Dr. Weil-Malherbe pointed out that with the oxidation procedure we use for dihydroxymandelic acid, dihydroxyphenylacetic acid yields the same end-product as does dihydroxymandelic acid, yet dihydroxyphenylacetic acid, when added in amounts normally present in urine and carried through the entire procedure, does not significantly increase the apparent values of dihydroxymandelic acid. We now use an anion exchange resin (Sandler), followed by a periodate oxidation (Pisano) for our determinations of 4-hydroxy-3-methoxymandelic acid (VMA). This, plus heating of the column eluate, eliminates in-

complete oxidation, which may be due to interfering substances in ethyl acetate and in some urines."

In reply to Weiner's inquiry about the periodate oxidation method of Anton and Sayre (Fed. Proc., **24:** 388, 1965) for metanephrine and normetanephrine, Weil-Malherbe stated that it could not be evaluated until the procedure has been published in detail.

CHENOWETH: "We have devised a quick method to reduce the fluorescence background of Al_2O_3 to nearly zero. Al_2O_3, 40/80 mesh, is placed in a convenient vertical tube and furnace system. Argon is caused to flow through vigorously enough to drive off the fine dust, while the furnace is brought to 1000°C. At this point, chlorine is introduced into the argon stream for 4 hr at this heat. During cooling, the argon stream continues."

SHARMAN: "Because of difficulties with the trihydroxyindole method, we have been estimating small amounts of dopamine by methods which involve the conversion of dopamine to a triacetyl derivative. The acetyl derivatives of catecholamines are easily formed in aqueous solution by the addition of acetic anhydride and sodium bicarbonate. They can be extracted into methylene dichloride and separated by paper chromatography, using solvent systems which have been developed for the separation of steroid compounds. We have found that a hardened, alkali-washed paper is most suitable, because it has a very low background fluorescence. When the acetyl derivatives are condensed with ethylene diamine they yield intensely fluorescent compounds. The colors of the fluorescence are such that triacetyl dopamine can be distinguished from the acetyl derivatives of most other catecholamines.

"Of the catecholamines examined, only the acetyl derivative of α-methyl dopamine gave a fluorescence similar to that of triacetyl dopamine, and these two compounds can be separated chromatographically. As the color of the fluorescence derived from triacetyl dopamine differs from the color of the blank fluorescence, one is able, by applying a set of simultaneous equations to measurements made at two different fluorescence wavelengths, to obtain an estimate of the blank fluorescence from the sample itself. In addition to increased specificity, there is a 3- to 4-fold increase in the intensity of the fluoresence developed, probably because of an increased solubility of the fluorescent product in isobutanol. It is possible to estimate and identify with reasonable certainty approximately 5 ng of dopamine. The method can be applied to the estimation of NE and E, but it will not distinguish between a catecholamine and its N-acetyl derivative when these occur together.

"The acetylation and condensation with ethylene diamine can also be applied to other methods of extracting dopamine; for example, we use this method to estimate dopamine in eluates from cation exchange resin columns, but we have no experience of using alumina as the adsorption medium (R. Laverty and D. F. Sharman: Brit. J. Pharmacol., **24:** 538, 1965)."

KOPIN: "I should like to assure Dr. Sandler that there are other methods for homovanillic acid which are specific and sensitive. The isotope-dilution technique of Weise *et al.* can be used for homovanillic acid, as this compound is eluted im-

mediately after VMA from the columns used in their assay. Mrs. E. Gordon and Miss E. Taylor at the N.I.M.H. have used isotopically labeled homovanillic acid simultaneously with labeled VMA to assay both of these compounds on the same columns."

GITLOW: "The VMA screening test whose usefulness was questioned by Dr. Sandler can be performed in a routine chemical laboratory and requires no columns, fluorimeter, or other special equipment. It requires about 2 hr to perform, and separates all urines which contain 4 μg VMA per mg creatinine from normals (*i.e.*, those containing 3.5 μg VMA or less per mg creatinine). No false negative results were found in a series of 49 patients with pheochromocytomas. The original test yielded false positive results (due to dietary indiscretion by the patient) in 10%, but a modified screening test yielded false positive results in only 0.9% of the normal cases. The Pheoset VMA technique is useless.

"Vanillin techniques for quantitative determination of VMA suffer from the drawback of high mean excretion levels in normal subjects. Since bidirectional paper chromotography yields mean VMA excretion levels under 2.5 mg per day, and recovery with this highly cumbersome technique is in excess of 95%, the vanillin spectrophotometric techniques yielding mean VMA excretion values in excess of 4 mg per day must be measuring substances other than VMA. This lack of specificity makes the use of such techniques for normal variation questionable. Small variations must not be evaluated by these tests.

"Both homovanillic acid and 3-methoxy, 4-hydroxyphenylglycol can also be assayed quantitatively by bidirectional paper chromatography, and the normal excretions of these substances are slightly less than that of VMA, rather than more, as reported with the use of various spectrophotometric techniques.

"We have successfully applied gas-liquid chromatography to the problem of VMA assay in urine, not so much because of the present inadequacy of VMA assay procedures, but more to serve as a model for planning more difficult procedures for the measurement of VMA in plasma, and other metabolites of catecholamines in urine and plasma. Present knowledge about the use of gas-liquid chromatography with halogenated acetyl derivatives and electron-capture detection promises remarkable sensitivity and specificity. A technique for measurement of plasma NE using gas-liquid chromatography seems more than likely in the near future."

HARRISON: "We have studied the mechanism of oxidation of catecholamines (*via* indoles) by various chemical and biologic agents and catalysts. The results are believed pertinent to methodology, and they may have biologic significance. Using fluorescence spectrometry, we have found that an oxidation intermediate, definitely not adrenochrome, is formed when E, in micromolar amounts, is oxidized in the presence of various metallo-proteins, metallo-enzymes, and free metal ions, *i.e.*, where O_2 is the electron- and proton-acceptor. It has been found (W. H. Harrison: Arch. Biochem. Biophys., **101:** 116, 1963) that the product of E 1) has characteristic fluorescence, 2) reduces ferricyanide with loss of fluorescence, the reaction being reversed on reduction (thus the intermediate is the reduced form of a reversible redox system), 3) is converted to a fluorescent

trihydroxyindole on alkalinization, and 4) does not form from adrenochrome or reduced adrenochrome. New unpublished work with Dr. Whisler, using tyrosinase as a catalyst in time-course studies, has shown that cyclization to a nonfluorescent intermediate occurs, followed by conversion to the fluorescent intermediate. The initial cyclization step was shown by following tritium released from the 6-position of ring-labeled E. Tautomerization did not occur, in that E labeled with tritium in the β-position of the side-chain did not lose tritium. NE, but not dopamine, forms a similar product with different fluorescence characteristics. The product is absorbed on alumina, whereas adrenochrome is not. We are characterizing the intermediate and determining the mechanism of this and similar oxidations."

Pekkarinen emphasized the importance of precision, simplification, and automation of methods in the fluorometric assay of E, NE, and their metabolites in large numbers of urine specimens. Among the modifications he suggested to these ends, as employed in his laboratory, are 1) prior washing of alumina with 10% Na_2HPO_4 to reduce blank values; 2) inclusion of ascorbic acid and EDTA for stabilization, and sodium citrate to prevent precipitation, with the sodium carbonate-bicarbonate buffer (pH 8.5) added prior to adsorption on alumina; 3) combination of potassium ferricyanide and secondary sodium phosphate in the oxidizing solution; 4) sodium bisulfite in combination with a small amount of ascorbic acid for tautomerization; after alkalinization and the initial reading, the same sample can then be shaken for an hour and used as the blank. He also described a micro-modification of Pisano's (1962) method for VMA, which employs only one-fifth the usual volume of reagents (Pekkarinen and Hakulinen, unpublished).

As the Chairman mentioned in her introductory remarks, a tremendous burst of activity within the past 3 years has resulted from the application of histofluorescent microscopic techniques for localizing catecholamines and certain other monoamines in nervous tissue. Among other things, it has perhaps provided the first reasonably convincing evidence of adrenergic transmission in the central nervous system. The technique is based on the extensive studies conducted by Eränkö (*e.g.*, Acta anat., **16:** suppl. 17, 1952) during the early 1950's, in which he showed that following the exposure of sections of the adrenal medulla to formaldehyde solution, there developed a fluorescence in NE-containing cells. The distribution of the fluorescence was carefully correlated with that of NE by the histochemical methods available at that time. These contributions have sometimes been overlooked. While the original method was insufficiently sensitive for localizing catecholamines in adrenergic nerve fibers, the means for doing this was described in the publications of Falck, Hillarp, and associates (J. Histochem. & Cytochem., **10:** 348, 1962; Acta physiol. scand., **56:** suppl. 197, 1962), in which freeze-dried tissues were exposed to formaldehyde vapor prior to sectioning. The wealth of new observations which the method has produced is amply illustrated by the reports at the present meeting.

In leading off the discussion of Owman's paper, Corrodi described the extensive studies by himself, Jonsson and associates on the chemistry, specificity, and ap-

plications of the method. A chemical treatment of fluorescing sections has been developed that permits differentiation between the reaction products of NE and dopamine; but *m*-tyramine and metaraminol form fluorescent products which are invisible inside the axon. (For a full presentation of this work, see H. Corrodi and N. Å. Hillarp, Helv. chim. acta, **46:** 2425, 1963; *ibid.*, **47:** 911, 1964; H. Corrodi, N. Å. Hillarp and G. Jonsson, J. Histochem. & Cytochem., **12:** 582, 1964; H. Corrodi, and G. Jonsson; Acta Histochem., in press; *ibid.*, J. Histochem. & Cytochem., in press; B. Csillik, J. Neurochem., **11:** 351, 1964; B. Falck *et al.*, J. Histochem. & Cytochem. **10:** 348, 1962; B. Hamberger *et al.*, J. Histochem. & Cytochem. **13:** 147, 1965; T. Malfors, Acta physiol. scand., **64:** suppl. 248, 1965; Ch. Sachs, Biochem. Pharmacol., in press).

Udenfriend's inquiry concerning the optical equipment available for such studies was answered by Hamberger, who related that for the measurement of small differences in emission and excitation spectra, equipment has been developed at the Institute of Cell Research of the Karolinska Institutet, Stockholm. This apparatus permits thorough estimation of spectra of both emission and excitation at a cellular level down to 0.5 μ. Some of Corrodi's experiments were performed with this equipment in cooperation with Dr. Ritzén, but it can no doubt be made with an ordinary microscope using proper filters.

The question of the sensitivity of the method, *i.e.*, the significance of the lack of detectable fluorescence in a given neuron, was raised by Salmoiraghi. Fuxe pointed out that while the possibility of the presence of subliminal concentrations could not be excluded, neurons that potentially contain monoamines can be identified by treatment with a monoamine oxidase-inhibitor and a precursor, which will produce fluorescence in the vast majority of special monoamine-containing structures. Similar studies were described by Langemann, who in collaboration with Lichtensteiger had treated mice with large doses of reserpine and nialamide, followed by NE, E, dopamine, or dopa. After NE, and to a less extent after the other three compounds, certain nerves and fibers in the hypothalamus and other regions of the brain showed a marked increase in fluorescence (J. Pharmacol., in press, 1965.)

In reference to Eränkö's paper, the question of the specificity of staining for acetylcholinesterase in the superior cervical ganglion cells of the rat was raised by Norberg and Sjöqvist. Norberg stated that he and Drs. Holmstedt and Hamberger had tested 18 different thiocholine esters as substrates with this ganglion, and had obtained staining with acetyl-, butyryl-, propionyl-, and valeryl-thiocholine. Eränkö pointed out the marked species difference between the rat and cat in this regard, which can account for the apparent discrepancies between his findings and those of the Stockholm group, who published only on the cat. Since neuronal staining with acetylthiocholine in the rat ganglion was obtained in the presence of 10^{-6} M iso-OMPA (a selective inhibitor of pseudocholinesterase) and nearly abolished by BW 284 C 50 (a selective inhibitor of acetylcholinesterase), it is reasonable to conclude that the enzyme responsible was chiefly acetylcholinesterase.

The latter viewpoint was also expressed by Koelle. While it is true that some

neurons in the rat superior cervical ganglion contain pseudocholinesterase, all appear to contain varying but significant concentrations of acetylcholinesterase on the basis of its resistance to low ($3 \cdot 10^{-8}$ M) and sensitivity to high (10^{-5} M) concentrations of di-isopropylphosphorofluoridate (G. B. Koelle, J. Comp. Neurol., **100:** 211, 1954). Likewise, in the rabbit and the rhesus monkey, in contrast to the cat, all sympathetic ganglion cells, and hence all adrenergic neurons, contain significant amounts of acetylcholinesterase; these studies were performed with complete biochemical controls (G. B. Koelle, J. Pharmacol., **114:** 167, 1955). The same difference between the cat and rabbit, *i.e.*, the absence or presence of acetylcholinesterase in adrenergic neurons, appears to apply also to most peripheral adrenergic fibers in these two species (D. Jacobowitz and G. B. Koelle, J. Pharmacol., **148:** 225, 1965).

Burnstock stated that whole mount preparations of the urinary bladder of the toad (*Bufo marinus*) had been stained for cholinesterase and catecholamines by his associates, Bell and McLean. Although both entities were noted in the large trunks, the fine branches going to the striated muscle bundles contain exclusively cholinesterase, whereas those innervating the arteries and arterioles show only catecholamine.

Weiner asked whether the presence of acetylcholinesterase is a valid criterion for identifying a neuron as "cholinergic." He pointed out that the correlation between the concentrations of acetylcholine and choline acetylase in various regions of the brain is much higher than that between acetylcholine and acetylcholinesterase. Koelle agreed with this, but called attention to the reasonably good parallel between choline acetylase and acetylcholinesterase concentrations in various tracts and nuclei of the cat brain, as published recently by Shute and Lewis (Nature, **199:** 1160, 1963). This question leads to the more general one of whether individual neurons are indeed exclusively cholinergic, adrenergic, or otherwise. According to more recent proposals, a given neuron may function with a combination of more than one transmitter (*e.g.*, J. H. Burn and M. J. Rand, Nature, **194:** 163, 1959; G. B. Koelle, J. Pharm. Pharmacol., **14:** 65, 1962). Rand illustrated this viewpoint by describing recent experiments in which both botulinum toxin and hemicholinium were shown to block the response of the piloerector muscles of the cat's tail to stimulation of the adrenergic excitatory fibers.

Davis mentioned some interesting observations in which central neurons which stain for acetylcholinesterase were found to be correspondingly sensitive to the microiontophoretic application of acetylcholine. However, as the chairman pointed out, the latter criterion would classify the neurons as "cholinoceptive" rather than "cholinergic." As a final remark, Feldberg emphasized the need for carefully controlled studies on normal and denervated ganglia before drawing firm conclusions regarding their contents of acetylcholinesterase or catecholamines.