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Chairman: Ulf S. von Euler, M.D.

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Preface

THIS Symposium on the "Regulation of Catecholamine Metabolism in the Sympathetic Nervous System" brings together just a few of the many investigators who have played important roles in the field of catecholamine research. The Symposium was designed to deal specifically with one important aspect of the sympathetic nervous system. Obviously, many other important areas of catecholamine research have also yielded exciting findings in recent years. It is hoped that a much larger and more comprehensive Symposium on Catecholamines, comparable to the one held in Milan in 1965, will be able to cover all major advances in the field since then. Such a catecholamine symposium is actually in the planning stage.

One of the people who played a key role in the organization of the 1965 symposium was Dr. Daniel Efron. Once again he helped get the planning for the new symposium organized and underway. It is with great sadness that we report his untimely death on January 27, 1972. Dr. Efron was a friend of all the participants in the present Symposium. In his position as Chief, Pharmacological Section, Psychopharmacology Research, National Institutes of Mental Health, he had a profound influence on all areas of neurobiology. We shall all miss him greatly.

The present Symposium owes its success to the superb organizational skills of the staff of the New York Heart Association, particularly Mrs. Ivane Saulpaugh and Mr. Charles Campbell.

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Molecular Biology of the Sympathetic Nervous System

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THIS Symposium, sponsored by the New York Heart Association, is most timely and demonstrates the rapid advances that have been made in our understanding of the sympathetic nervous system. The discovery of chemical neurotransmitters was made early in this century but the identification of the sympathetic neurotransmitter, norepinephrine, was made only a little over 20 years ago. Its precursors and metabolites were identified even more recently. The enzymes involved in the biosynthesis and metabolism of the transmitter have received intensive study and most of them have now been purified and characterized. However, all this is not enough to tell us how the sympathetic nervous system functions. Some hints of regulatory processes in the sympathetic nervous system were presented at the Second Catecholamine Symposium in 1965 (3). Since then we have begun to recognize that sympathetic nervous activity is modulated by changes in the rate of synthesis and degradation of dopamine, norepinephrine and epinephrine. Regulation by end-product inhibition, allosterism, repression, and derepression, mechanisms which were first established in studies on microorganisms, are now known to play important roles in the sympathetic nervous system. We are also beginning to recognize the significance of the organization of the enzymes within the nerve cells with respect to their regulation and function. The participants of this Symposium will discuss current concepts of regulatory mechanisms in the sympathetic nervous system and present some of the experimental evidence on which these concepts are based. It will become evident during these discussions that newer technology, much of it derived from other fields such as immunology, fluorescence and electron microscopy, make possible these advances. Clinicians as well as laboratory scientists are now aware that the turnover of the catecholamines in tissues is of greater significance than the observed tissue concentrations.

It will also become apparent during this Symposium that we are no longer talking of one transmitter. Furthermore changes in nerve activity, produced by whatever mechanisms, result in changes in the rates of synthesis of these transmitters. Acute changes in nerve activity trigger one type of mechanism (1); chronic changes in nerve activity trigger another type (2, 4). Thus during short periods of exercise, hypotension (drug induced or endogenous) or decreased temperature, norepinephrine synthesis is increased. The onset of this type of increased synthesis is extremely rapid as is its disappearance when conditions revert to normal. Under such conditions no changes are observed in the amounts of synthetic enzymes in the tissues. When great demands are made on the sympa-

thetic nervous system, over long periods of time it responds by gradually increasing the levels of catecholamine synthesizing enzymes in the tissues. Conversely, in hypertension or under conditions where there is an increased production of catecholamines, levels of the synthetic enzymes in tissues may be diminished.

It has also been apparent for a long time that other hormones modify sympathetic activity. Some of these interactions can now be explained at the enzymatic level.

These newly discovered mechanisms may explain observed physiological and pathological responses. They may also explain the actions and limitations of some present day drugs and may make possible the development of new drugs. Although such new information is important in itself, it is obvious that it also has great practical value in clinical medicine. One can expect from it new advances in diagnostic and therapeutic procedures, particularly in cardiology, neurology and psychiatry.

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Increased Synthesis of Catecholamines without Changes in Enzyme Levels

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Dopamine and Norepinephrine in Noradrenergic Axons: A Study *in Vivo* of Their Precursor Product Relationship by Mass Fragmentography and Radiochemistry

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Introduction

THE localization of catecholamines in cell bodies, axons, and axon terminals of sympathetic neurons is not random but is regulated by a set of chemical laws which are still poorly understood. We know that tissue catecholamines can be extracted as distinct molecules by simple homogenization in acidic media. This suggests that covalent bonding cannot be involved; if catecholamines were united by covalent bonds to each other or to other molecules while stored in neurons, their extraction in cold perchloric acid 0.4 M would be impossible. We know that the concentration of catecholamines stored in specialized subcellular structures (synaptic vesicles) is hypertonic (23, 38) and this brings up the possibility that bonds weaker than covalent bonds are involved in catecholamine storage (23). Although in histochemical analysis (16, 17) after fixation and dehydration the norepinephrine (NE) localization appears rather predictable, the nature of the NE binding in the vesicles (33) and other kinetic considerations (10–26) suggests that a dynamic state may be involved in NE storage. Usually, it is postulated that the NE stored in synaptic vesicles exchanges with other neuronal sites at a very slow rate (36). Actually, current views tend to describe extraneuronal release of catecholamines by nerve impulses as involving a small metabolic pool of transmitter (24, 27) which eludes histochemical localization. Now that we are acquiring data compatible with the release of NE from sympathetic nerves by a process of exocytosis (15, 18, 19, 40), the view that newly synthesized NE should be preferentially released by nerve impulses cannot be readily explained. However, several simple models of adrenergic function include two pools of catecholamines, respectively termed storage and metabolic, which function as two distinct enti-

ties and mix with each other at a very slow rate (4, 24, 35). Pletscher *et al.* (33) suggested that van der Waals' forces are involved in determining the localization of catecholamines in subcellular particles. If catecholamine binding in synaptic vesicles involves van der Waals' forces (33), we can surmise that the energy requirement may not be greater than 6 kcal/mole. It is appropriate to keep in mind that this energy is only about 10 times larger than the average energy of heat motion at 25°C (0.6 kcal/mole). This suggests that at physiological temperatures, various neuronal compartments may include many NE molecules with sufficient kinetic energy to break van der Waals' forces. Therefore, it appears conceivable that the average life time of one of these weak bonds can be estimated in the order of a fraction of a second. This reaction rate is so rapid that neurons do not need enzymes to speed up the rate of association and dissociation of these weak bonds. If we assume that van der Waals' forces function in the storage of catecholamines in synaptic vesicles, a NE molecule, while confined in this compartment by physiological and physicochemical properties of the membranes, may interact several times in a minute with other catecholamine molecules as well as with other components of the storage vesicles, including adenosine triphosphate (ATP). Obviously, these interactions increase the level of probability that a certain degree of NE mixing might occur while the amine is stored. Mixing of axonal NE involves the continuous motion of NE molecules within a compartment but also their passage from one compartment to another and their release into and their uptake from extraneuronal spaces (28). Ultimately, we must consider that NE turnover can proceed at variable rates (7, 11); these are reflected in the renovation rate of the 3,4-dihydroxyphenylethylamine (DM) of noradrenergic neurons because this amine is the obligatory and immediate precursor of NE. DM, which is synthesized by a soluble enzyme (L-aromatic acid decarboxylase EC 4.1.1.26) (5), may not be localized in a specific neuronal site but freely distributed in the cytosol. There it forms NE by interacting with dopamine-β-hydroxylase (EC 1.14.2.1), an enzyme which is either bound to synaptic vesicle membranes (34) or is soluble within this storage particle (39).

The regulation of catecholamine synthesis is complex and depends also on the rate of axonal depolarization (13, 30, 32). It is this relationship between turnover rate of catecholamines and neuronal activity that has directed the interest of many investigators to calculate turnover rate of NE in intact tissues (9, 13, 29). Implicit in this interest is the hope that the measurement of NE turnover rate might be an index of electrical activity in a given population of noradrenergic neurons and therefore might facilitate the study of the localization of drug actions in the central nervous system. However, we must remember that turnover rate changes elicited by drugs do not reflect exclusively their action on the rates of neuronal activity. A drug may affect turnover rates of catecholamines by interacting with enzyme activities related to catecholamine metabolism or it may interfere with other mechanisms that regulate turnover (29). It follows that in evaluating drug mechanisms, the measurement of catecholamine turnover rate must be tempered by other biochemical studies. By measuring only turnover rates, one cannot conclude that the drugs which change this parameter also alter

the rate of depolarization of noradrenergic neurons. This presentation deals with our latest attempts to test the validity of previous methods of measuring NE turnover rate *in vivo* (11, 12, 14, 30, 31). We report on the kinetics of the precursor-product relationship between DM and NE measured in rat heart ventricles, a tissue containing noradrenergic, but not dopaminergic, axons (1, 37).

Models to Measure Turnover Rate of Catecholamines

Several attempts have been made to describe catecholamine compartmentation by suitable mathematical models (10, 36). However, as discussed by Bergman (3), it is difficult to formulate an exact model that appropriately weighs the numerous factors involved in controlling the steady-state of endogenous chemicals functioning in a biological system. Usually, the biological system is not fully understood; the measurements include a population of compartments, rather than a single compartment; the number of measurements that can be performed in the same sample is often limited to one, and the uncertainty involved in these measurements is usually quite large. Up to now these complications have frustrated any attempts to describe the regulation of NE turnover with a satisfactory mathematical model. As an alternative, a number of simplifying assumptions have been made which, in turn, have led to the formulation of a simple, but probably inexact model of noradrenergic function (10–14). These models are tested and the results are used to modify and improve the model. Since the coincidence of experimental results obtained with different methods to measure NE turnover gives relevance to the basic assumptions, an operational device often followed is that of parallel experimentation with various methods to measure NE turnover rate (12). Kinetically, we have viewed (30, 31) neuronal catecholamines as if they were stored in a compartment open at both ends

$$A \xrightarrow{k_1} B \xrightarrow{k_2} M \qquad \text{(model } a\text{)}$$

In model *a* the compartment of tyrosine, the catecholamine precursor at the rate limiting step, is indicated with A; B represents the catecholamine compartment; and M, a generalized metabolic compartment including the various catecholamine metabolites produced either intraneuronally or extraneuronally. Since this process is irreversible and the NE compartment is kept at steady-state, metabolism represents a net loss of catecholamines from compartment B, regardless of whether the enzymatic alteration occurs intraneuronally or extraneuronally. The rate of this loss is an important kinetic parameter and it is described by the product k_2B. Although NE turnover rate fluctuates rapidly and continuously, we assume that within the time constant of our sampling, the NE compartment remains at steady-state; hence k_2B is balanced by the rate of catecholamine formation which in the above scheme is described by k_1A. Neither k_1 nor A can be measured directly since in brain and other tissues tyrosine is also a precursor for many other metabolic processes, there is no way to physically separate the various metabolic pathways involving tyrosine. As an alternative, we have related the changes with time of tyrosine and NE specific radioactivity and from this relationship we have

TABLE 1

Fractional rate constant for NE efflux from rat brain

Method	k hr^{-1}	Reference
Blockade of synthesis	0.17	6
Labeling with ^{14}C-tyrosine (constant rate of i.v. infusion)	0.25	30
Labeling with 3,5-^{3}H-tyrosine (pulse i.v. injection)	0.28	31

estimated k_2, since B can be measured, the NE turnover rate can be estimated. This and other estimations of NE turnover rate are summarized in the following pages. The reader interested in a detailed description of these methods should refer to previous publications from our laboratory (9, 10, 13, 14, 30, 31).

Methods to Calculate Catecholamine Turnover Rate in Vivo

In model *a* the two fractional rate constants, k_1 and k_2, describe the fraction of A and B that per unit of time is changing its chemical identity in the direction indicated by arrows. The validity of this scheme for measuring the fractional rate constant of brain NE efflux has resisted a number of parallel tests summarized in table 1. The results reported in this table suggest that by using isotopic and non-isotopic methods and assuming that NE is stored in a single compartment open at both ends, we have obtained similar values of k_2 although each method listed in table 1 involves a slightly different set of underlying assumptions. The calculation of k_2 after instantaneous blockade of NE synthesis (6, 12) takes advantage of the finding that in rats the concentration of brain NE declines as a single exponential for several hours if the inhibitor of NE synthesis is injected intravenously twice in the doses of 200 and 75 mg/kg at time 0 and 2 hr, respectively. These results indicate that the efflux of NE is proportional to the NE concentrations present at any time. Since at steady-state, synthesis and efflux rates are identical, it follows

$$T_R = k_2[NE]_0 \qquad I$$

Where T_R is the turnover rate of NE, k_2 is the fractional rate constant of NE efflux and $[NE]_0$ is the brain concentration of NE at physiological steady-state. Since at steady-state

$$\frac{-d[NE]}{dt} = k_2[NE]_0 \qquad II$$

integrating

$$[NE] = [NE]_0 e^{-k_2 t} \qquad III$$

and converting to $\log_{10}$

$$\log [NE] = \log [NE]_0 - 0.434\, k_2 t \qquad IV$$

Equation IV shows that the slope of the decline of log NE concentrations after blockade of synthesis is 0.434 times k_2. The theoretical relationship guiding estimation of synthesis rates of any chemical constituent of animal tissues by precursor labeling, shows that the specific activity of the product (SB) changes with time in proportion to (a) the specific activity of the precursor (SA), and (b) the turnover rate and metabolism of the product (B). This basic tenet can be expressed by equation V where B* represents the abundance of radioactive molecules in B.

$$T_R = \frac{B^*_{t2} - B^*_{t1}}{SA\Delta_t} \qquad V$$

Equation V which is the common form used for calculating the "radiochemical yield" of a reaction, assumes that in this system SA (A*/A) is constant and that the rate of metabolism of B is close to zero during Δt. In practice, Equation V tells us that to measure the turnover rate of a compound *in vivo* we must know how B* changes with time and we must measure SA. But we know that in the case of turnover rate measurement of tissue NE, the amine is continuously made and metabolized and that SA in tissues changes at various times, after intravenous injection of tyrosine unless we have adopted some device to keep the tyrosine SA constant. Indirectly, these considerations underline the approximation of many studies where the turnover rate of NE or DM is estimated by measuring only B*/B at one time after the injection of A*. In practice, B* is expected to change as indicated in equation VI.

$$\frac{d\,B^*}{dt} = k_1\,A^* - k_2\,B^* \qquad VI$$

where k_1 is the fractional rate constant for the change of the label in A, and k_2 that for the change of the label in B. In our model, A represents tyrosine and B represents NE, both compartments are at steady-state and, therefore, k_1 and k_2 describe the efflux of the two compounds when calculations are made in terms of unit radioactivity

$$\frac{d\,SB}{dt} = \frac{k_1\,A^*}{B} - k_2\,SB \qquad VII$$

but at steady-state

$$k_1\,A = k_2\,B \quad \text{or} \quad B = \frac{k_1 A}{k_2} \qquad VIII$$

substituting in VII

$$\frac{d\,SB}{dt} = k_2(SA - SB) \qquad IX$$

We have published (31) the derivation of equation IX showing that k_2 can be

approximated as in equation X.

$$k_2 = \frac{2(SB_{t2} - SB_{t1})}{\Delta_t[(SA - SB)_{t2} + (SA - SB)_{t1}]} \qquad X$$

In practice, to measure the k_2 of brain NE stores a group of rats is given intravenous injections with a tracer dose of 3,5-^{3}H-L-tyrosine (1 mCi/kg; 30 Ci/mmole) and the specific activities of tyrosine and NE in brain are measured at various times after the injection of the label. The data are plotted on semilogarithmic paper against time and the k_2 is calculated with equation X from experimental points read on the graph at various time intervals. The validity of this estimation depends very much on the ability of measuring accurately the value of SA at various times after labeling. Since tissue tyrosine not only is the precursor of catecholamines at the rate limiting step but also is involved in many other metabolic processes of neurons, one cannot safely assume that in a given tissue the various tyrosine compartments reach an identical specific activity after the pulse injection of radioactive tyrosine.

The data reported in table 2 show that when equation X is applied to measure k_2 of the NE efflux from heart ventricles of rats receiving a pulse injection of 3,5-^{3}H-tyrosine the results obtained are at variance with the values calculated with three other methods. These include labeling with ^{14}C-tyrosine (30) by intravenous infusion at constant rate. We report in figure 1 the changes of SA (tyrosine in heart ventricles) and SB (NE in the same tissue) after pulse injection of 3,5-^{3}H-tyrosine. The data of figure 1 show that a rather consistent value of k_2 is obtained by applying equation X to the data point read from the graph for successive 0.03-hr time intervals. However, the mean value of k_2 shown in figure 1 is at variance with the results obtained with other methods (table 2). From these results we infer that some of the basic assumptions adopted to measure NE turnover rate in rat heart ventricles after pulse injection of ^{3}H-tyrosine are perhaps unjustified. This discrepancy cannot be attributed to the short time interval after the pulse injection of tyrosine we have selected to perform the measurement of SA and SB, since in the experiments with constant rate intravenous infusion of ^{14}C-tyrosine (table 2) our measurements were also taken during 0.6 hr from the beginning of the labeling (30). To elucidate this discrepancy, we decided to consider whether the rate of injection influenced

TABLE 2

Fractional rate constant for NE efflux from rat heart ventricles

Method	k hr^{-1}	Reference
Uptake of L-^{3}H-NE	0.076	6
Blockade of synthesis	0.076	6
Labeling with ^{14}C-tyrosine (constant rate i.v. infusion)	0.089	30
Labeling with 3,5-^{3}H-tyrosine (pulse injection)	0.52	Present paper