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Increased Synthesis of Catecholamines without Changes in Enzyme Levels

CHAIRMAN: SEYMOUR S. KETY, M.D.

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

E. COSTA, A. R. GREEN, S. H. KOSLOW, H. F. LEFEVRE, A. V. REVUELTA AND C. WANG

Laboratory of Preclinical Pharmacology, National Institute of Mental Health, Saint Elizabeths Hospital, Washington, D.C.

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

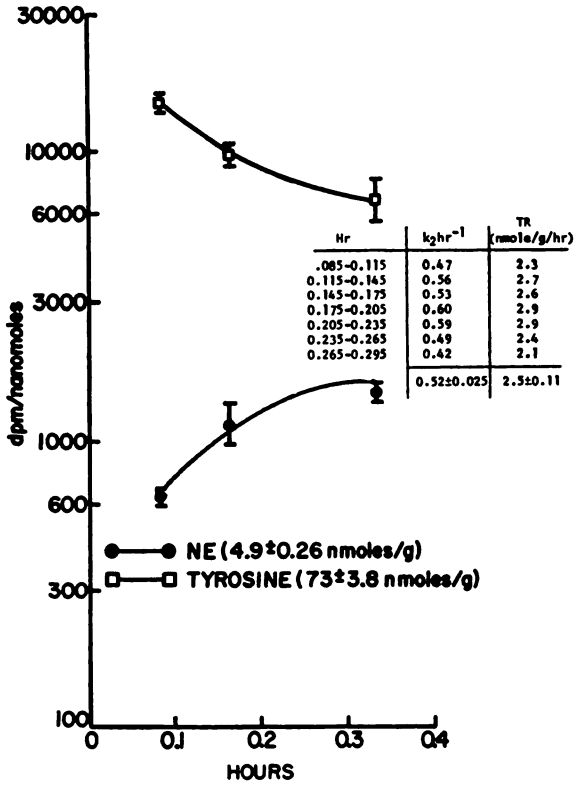


FIG. 1. Time course of the specific activity of tyrosine and NE in heart ventricles of rats receiving at 0 time 1.5 mCi/kg intravenously of 3,5-^{3}H-tyrosine (S.A. 30 Ci/mmole). In the figure, the values of k_2 calculated (according to equation X) for successive 0.03-hr intervals are also shown.

the form taken by the curve describing the change with time of the specific activity of tissue tyrosine in various tissue compartments.

Equation XI describes the changes of tissue tyrosine specific activity after radioactive tyrosine injections

$$\frac{d\,SA}{dt} = K - k_1\,SA \qquad XI$$

k_1 is the apparent rate of change of SA, in this case SA refers to the specific activity of tyrosine in the plasma compartment and K is the rate of entry of tyrosine in this compartment. Imposing the condition that SA = 0 at t = 0 and integrating

$$SA = \frac{K}{k_1}(1 - e^{-k_1 t}) \qquad XII$$

Substituting SA in equation IX with the value of SA expressed in XII and in-

tegrating with the constraint that at t = 0 SB = 0

$$SB = \frac{K}{k_1}\left[1 + \frac{1}{k_2 k_1}(k_1 e^{-k_2 t} - k_2 e^{-k_1 t})\right] \qquad \text{XIII}$$

With equation XIII, the value of SB could be calculated with a sequence of theoretical values of k_2 (30). Solution of equation XIII is possible under this condition because the values of K and k_1 either are known or can be obtained from equation XII. These arbitrary solutions of XIII allow us to express graphically the relationship between SB and k_2. By plotting in a cartesian graph the sequence of the theoretical values of k_2, we have selected to obtain various solutions of equation XIII against the value of SB calculated for each theoretical value of k_2, a graph can be constructed where to each value of SB corresponds a value of k_2 (30). Obviously, this relationship is valid only for a given rate of ^{14}C-tyrosine infusion and for a given time period of infusion because these two parameters determine the values of K and k_1. Now by replicating the rate and duration of precursor infusion whereby the values reported in the graph were obtained, one can estimate k_2 by reading off the graph the value of k_2 which corresponds to any experimental value of SB (30).

Problems Related to Measurements of NE Turnover in Rat Heart Ventricles by Pulse Labeling with ^{3}H-Tyrosine

Since the discrepancy of the k_2 value for NE efflux calculated after pulse injection of ^{3}H-tyrosine was marked in heart ventricles (table 2) but was less evident in brain (table 1), we decided to use the rat heart ventricles to study the cause for such a discrepancy. Indeed, the model of a single compartment open at both ends approximates rather than describes the dynamics regulating NE steady-state in sympathetic nerves. The following should be a more realistic model

$$\begin{array}{l} A(TY) \xrightarrow{k_1'} A'\,(dopa) \xrightarrow{k_1''} A''\,(DM) \xrightarrow{k_1'''} B\,(NE) \xrightarrow{k_2} M \\ \quad\downarrow \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \text{(model } b\text{)} \\ \quad X \end{array}$$

k_1

$k_{TY_1}\,;\, k_{TY_2}\,;\, \cdots\, k_{TY_n}$

Clearly model *b* shows that in the simplified model *a* k_1 includes a number of fractional rate constants: two for the biosynthesis of the obligatory precursors of NE, 3,4-dihydroxyphenylalanine and 3,4-dihydroxyphenylethylamine (DM) and a number of k_{TY} (k_{TY_n}) describing other metabolic pathways of tyrosine. It is possible that in harmony with the assumptions made in the simplified model, the specific activity of the various tyrosine pools after pulse labeling reaches the maximum shortly after 0 time; however, it is unlikely that the specific activity of these tyrosine pools is uniform. The discrepancy in the SA of total tissue tyrosine and that of the tyrosine compartment functioning as a precursor to NE may be emphasized by labeling with pulse injections of tyro-

sine. In this method the specific activity of plasma tyrosine increases abruptly, thus amplifying any possible differences in the turnover rate and consequently in the specific activity of the various tyrosine pools. Since in several species (1, 37) including the rat (table 6) the DM pool of heart ventricles is a few percent of the NE pool and is probably located in noradrenergic nerves where it functions as the obligatory precursor to NE, this DM pool should equilibrate rapidly with the tyrosine pool involved in NE biosynthesis. We reasoned that by measuring simultaneously the specific radioactivity of tyrosine, DM and NE in the heart ventricles of rats receiving a pulse injection of 3,5-^{3}H-tyrosine we might verify whether various tyrosine pools in rat heart ventricle have different specific activities. Moreover, since DM is the immediate obligatory precursor of NE, its turnover rate would give us a good estimate of the absolute synthesis rate of NE in rat heart ventricle. We overcame the inherent difficulty of assaying with absolute specificity the small concentrations of DM present in heart ventricles of rat by using the mass fragmentographic method developed in our laboratory (8, 27).

Mass Fragmentographic Assay of Catecholamines

Electron impact mass spectrometry (MS) identifies molecules by recording the mass/charge (m/e) value of the various positively charged fragments which are generated when a molecule undergoes electron impact at a pressure of about 10^{-6} atm. The magnetic deflection MS used in our experiments (LKB 9000 GC-MS) separates the various fragments by accelerating them with a constant potential and deflecting them within a flight tube passing through a magnetic field of variable strength. Equation XIV describes how m/e values are related to magnetic field strength (H) and accelerating voltage (V).

$$m/e = \frac{H^2 r^2}{2V} \qquad \text{XIV}$$

r is a constant indicating the radius of the curvature of the ion beam in the flight tube. Positive molecular fragments of any m/e value within the mass range of the instrument can be focused through the ion exit slit by appropriately changing the magnetic field strength (H). In practice, to obtain the mass spectrum of a compound the accelerating potential is kept constant and the magnetic field strength is increased at an exponential rate. Any given value of the magnetic field strength deflects the molecular fragments of a specific m/e value present in the accelerated ion beam to pass through the ion exit slit at the end of the flight tube and to impinge on the ion collector. A mass spectrum of a compound can be recorded if 10^{-6} to 10^{-7} moles are available. Equation XIV shows that when H^2 and V are kept constant, only a population of molecular fragments characterized by a given m/e value will be detected. By keeping the magnetic field strength constant the sensitivity of the detector can be increased by about 10^7 fold when the MS is used as a detector of a gas chromatograph (GC) in the LKB 9000 GC-MS. This is possible because the ion density generated by a specific fragment can be continually monitored and recorded

during the elution time of the compound from the GC column. At this high level of sensitivity, absolute specificity of qualitative identification can be obtained by simultaneously monitoring two or more fragments (multiple ion detection, MID) of the compound. Since the ratio of the ion density generated by various fragments of a molecule is a characteristic and unique property expressed by the mass spectrum of a molecule, MID simultaneously gives valid structure and quantitative information. The LKB 9000 GC-MS used in these experiments is equipped with an accelerating voltage alternator (AVA) which allows for the continuous monitoring of the ion density of two or three positively charged fragments of the parent compound. This device is used in conjunction with a constant magnetic field strength and allows a MID analysis of fragments with m/e values within 10 % of each other. The combination of GC-MS and MID as a method for identification of compounds in tissue specimens has been termed mass fragmentography by Hammar *et al.* (21). The reader interested in details of mass fragmentography is invited to consult a review on the subject published by Hammar *et al.* (22). In brief, identification of a compound is based on its GC retention time and the measurement of the ratio between the ion densities generated by two or three characteristic fragments (MID) of the compound; quantification is obtained by measuring the ion density generated by a characteristic fragment (usually the most abundant) at the specific retention time of the compound. The ratio between the ion density generated by a fragment of the unknown and that generated by a fragment of the internal standard added to the tissue sample before processing it allows quantification when compared to standard curves obtained by keeping the internal standard constant and changing the amounts of the authentic amine to be analyzed in the tissue.

For exact details of the method the reader is advised to consult the publications by Cattabeni *et al.* (8) and Koslow *et al.* (28). In brief, for GC separation the catecholamines are reacted at 60°C, 30 min with pentafluoropropionic anhydride to form acylated derivatives (PFP) with a higher vapor pressure than the parent compounds. As internal standards we used α-methylnorepinephrine (α-MNE) an α-methyldopamine (α-MDM) for NE and DM, respectively. These α-methyl analogues are used as internal standards since they fulfill the following requirements: (a) the forward and backward rates of acylation are identical to those of the catecholamines; (b) the acylated derivatives have a GC retention time different from that of the catecholamine; and (c) their fragmentation pattern in MS is similar to that of the catecholamine.

An abridged mass spectrum of NE-PFP, E-PFP and DM-PFP is presented in figure 2. It can be seen that these compounds have their most abundant fragments at m/e 176, 190 and 428, respectively. The mass spectra for α-MNE-PFP and α-MDM-PFP have been previously published (28). The structure of NE-PFP and DM-PFP fragments used for MID are reported in tables 3 and 4. These tables report the relative intensity of these fragments, their m/e value and the ratio between m/e value of the pairs of fragments used for the identification of these compounds by MID. Table 5 reports the GC retention time and

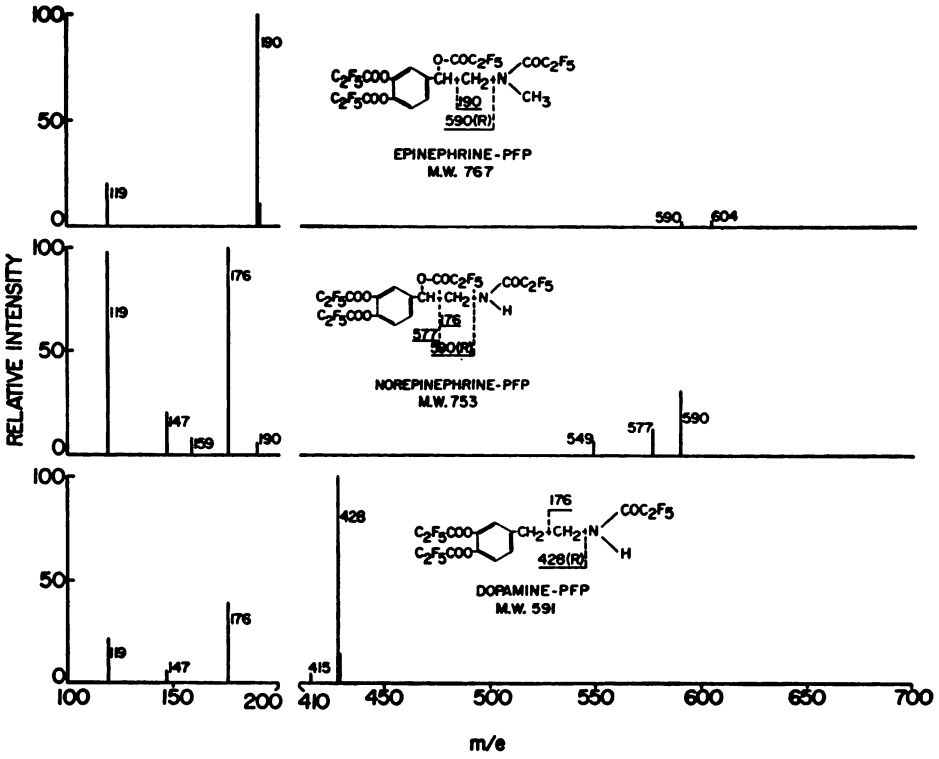
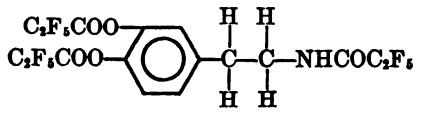


FIG. 2. Salient parts of the mass spectrum of E-PFP, NE-PFP and DM-PFP. The base peak of NE-PFP m/e 176 is not present in the mass spectrum of E-PFP. This peak allows for differentiation of these two compounds which have similar GC retention time. The fragments m/e 577 and 549 are unique for NE-PFP while 428 and 415 are unique for DM-PFP. These fragments are used for MID identification of the two compounds.

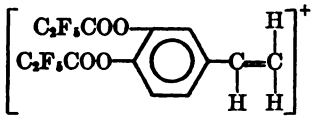
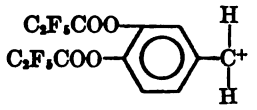
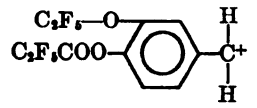
salient mass spectral characteristics of E-PFP, NE-PFP and DM-PFP and their internal standards. Only E-PFP and NE-PFP have identical retention times in our experimental conditions; however, E-PFP has no fragment at m/e 176, which is the base peak for NE-PFP and was used routinely for the assay reported in this paper. Moreover, we verified linearity with the ion density ratio generated by the base peaks of NE-PFP and DM-PFP and that of the appropriate internal standard peaks (*e.g.*, NE-PFP/α-MNE-PFP). Measurements of ion densities generated by the base peaks of NE-PFP and DM-PFP at their GC retention times therefore provide an extremely sensitive and specific analytical method for NE and DM concentrations in tissues. The linearity of the reaction of catecholamines and their internal standards with PFPA was shown by holding the concentration of the internal standards constant (20 pmoles) and varying the concentration of NE and DM from 0.5 to 200 pmoles (8, 28). This linearity applies to endogenous catecholamines (8, 28).

TABLE 3

Mass fragmentographic identification of dopamine PFP



mw = 591

Fragment structure	Relative intensity	m/e	Ratio
	100	428	
	5	415	20
	7	387	1.4
			14

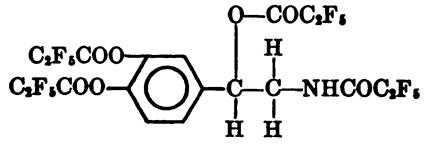
The loss of CO by molecular elimination from fragment with m/e of 387 might occur from any of the acylated side chains.

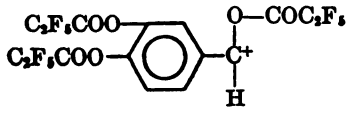
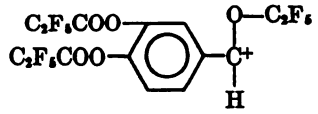
Mass Fragmentographic Assay of Heart Ventricle Catecholamine Concentration

In the present experiments, we analyzed the specific radioactivity of DM and NE in heart ventricles. At various times after injecting 1.5 mCi/kg of 3,5-^{3}H-tyrosine (SA 30 Ci/mmole) into the tail vein of Sprague-Dawley male rats (200 g body weight), the animals were killed and the heart ventricles dissected and immediately frozen. At a later time, the tissues were homogenized in 0.4 N perchloric acid containing sodium metabisulfite (0.5 mg/ml) and were centrifuged at 4°C (15,000 × *g* for 15 min). The supernatant was brought to pH 8 with Tris buffer and adsorbed in Al_2O_3 (500 mg). The alumina was washed with H_2O and the catechols eluted into 5 ml of 0.4 M formic acid. DM and NE can be assayed by mass fragmentography either in this eluate or in the second Al_2O_3 eluate where the radioactivity was routinely measured. To measure the radioactivity, the first Al_2O_3 eluate was reacted with CH_3COOK (10 M) and brought to pH 4.5 before proceeding to the DM and NE purification by ion exchange chromatography (Dowex 50 W H^+ × 4, 200–400 mesh) and a second Al_2O_3 adsorption as described previously (31). Mass fragmentographic assay of NE and DM was performed by focusing the GC-MS sequentially at the following m/e values: α-MNE, 190; NE, 176; α-MDM, 442; DM, 428, and the ion densities recorded. Absolute identification of DM and NE was obtained by

TABLE 4

Mass fragmentographic identification of norepinephrine PFP



Fragment structure	Relative intensity	m/e	Ratio
	12	577	1.71
	7	549	

The loss of CO by molecular elimination from fragment with m/e of 549 might occur from any of the acylated side chains.

TABLE 5

GC-MS characteristics of the pentafluoropropionyl (PFP) derivatives of norepinephrine, dopamine and their α-methyl analogues

PFP Amine	GC Retention	Relative Intensity (%)							
		176	190	415	428	442	577	590	604
	min								
α-MNE	2.08		100*						10†
NE	2.83	100*					12‡	30†	
E	2.83		100*					1.8†	2.5
α-MDM	4.36		100*			33†			
DM	5.83	40*		5‡	100†				

* Cleavage between α and β C. Positive charge is retained on the N containing fragment.

† Rearrangement and cleavage between N and α C. Positive charge is retained on the fragment containing the aromatic ring and one H is transferred onto the neutral moiety.

‡ Cleavage between α and β C. Positive charge is retained in the fragment containing the aromatic ring.

focusing on the pair of fragments listed in tables 3 and 4. GC retention time, continuous recording of the ion densities at two specific m/e values and measurement of the ion densities ratios establish absolute identity of the compounds. Figure 3 shows an example of the MID of NE and DM extracted from rat heart ventricle. It may be pertinent to note that despite the close similarity between the GC properties of NE and E (table 5) MID allows for a net distinction. In fact, the base peak of NE-PFP with a m/e value 176 and the fragments with m/e value of 577 and 549 are not present in the mass spectrum of

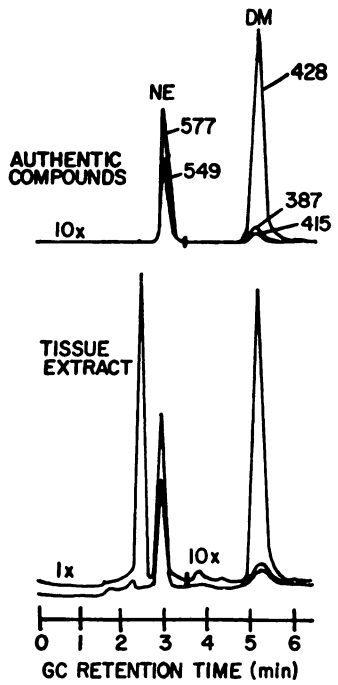


FIG. 3. Identification by multiple ion detection (MID) of rat heart ventricle NE and DM. Please note the identity of the ion density ratios of the various peaks used for MID identification in authentic compounds and in compounds found in tissue extracts.

E-PFP. Conversely, the fragment characterized by m/e value of 604 is absent in the mass spectrum of NE-PFP and can be used to identify E-PFP. A comparison of tissue catecholamines assay with fluorescence spectrometry and mass fragmentography shows that the two methods yield comparable results (table 6).

DM and NE Precursor Product Relationship in Noradrenergic Nerves of Rat Heart Ventricles

Angelakos *et al.* (1) have studied the distribution of E, NE, and DM in various parts of the rabbit and guinea pig heart and found that in ventricles, E and DM represent 4% and 2% of the amount of NE stored. More recently, Shahab *et al.* (37) have shown that DM cannot effectively utilize the NE binding site of the rabbit heart. These findings and other evidence discussed previously (8) support the view that DM present in heart ventricles represents a DM pool in noradrenergic nerves, presumably a DM pool functioning as a precursor of NE. After pulse labeling with 3,5-^{3}H-tyrosine, we attempted to calculate the efflux rate of NE from rat heart ventricle by measuring the changes with time of the specific radioactivity of DM and NE. By referring to the model *b*, we assume that the DM pool in heart ventricles resides in noradrenergic axons and functions as the obligatory and immediate precursor of NE.

TABLE 6

Assay by mass fragmentography and spectrofluorometry of norepinephrine (NE) and dopamine (DM) concentrations in rat tissue

Tissue	Mass Fragmentography			Spectrofluorometry		
	Tissue analyzed (mg)	NE	DM	Tissue analyzed (mg)	NE	DM
	nanomoles/g ± S.E.M.					
Striatum	27	2.0 ± 0.074	81 ± 5.2	68	*	63 ± 3.3
Cerebellum	250	1.1 ± 0.26	0.21 ± 0.04	250	0.83 ± 0.076	*
Vas deferens	41	130 ± 3.9	1.6 ± 0.2	41	110 ± 1.0	*
Cervical sympathetic ganglion	1.4	78 ± 6.1	13 ± 1.7	4	*	*
Heart ventricles	14.5	2.1 ± 0.073	0.29 ± 0.009	1500	2.6 ± 0.26	0.21 ± 0.09

* Undetectable in this amount of tissue.

In figure 4 we have plotted the change with time of DM and NE specific radioactivity found in heart ventricles of rat injected with 1.5 mCi/kg intravenously of 3,5-^{3}H-tyrosine (SA 30 Ci/mmole). Calculation of k_2 according to equation X shows that this value is definitely smaller than that calculated from the data reported in figure 1.

The k_2 value calculated from DM and NE specific activity (fig. 4) is similar to those reported in table 2, which were calculated after either blockade of synthesis or labeling with pulse injection of L-^{3}H-NE or constant rate infusion of ^{14}C-tyrosine. With the k_2 value calculated from the data reported in figure 4, the turnover of rat heart ventricle NE is estimated equal to 0.69 nanomoles/g/hr. If the assumption that DM in heart ventricles is the obligatory precursor of NE is accepted, then this turnover rate value can be taken as a measure of absolute synthesis rates of heart ventricle NE. As we mentioned previously, the finding that this value differs from that calculated from the changes with time of the specific activity of rat heart ventricle tyrosine and NE, may indicate that our simplified single compartment model may not be consistent with the actual compartmentation of heart ventricle tyrosine as it relates to NE turnover rate measurements. This reasoning prompted us to consider a mathematical model with a multiple compartmentation of tyrosine, to verify whether by using changes of DM specific radioactivity, we could identify and characterize kinetically the compartment of heart tyrosine which is involved in the biosynthesis of NE. Moreover, the kinetic parameters of this tyrosine compartment would give us additional information to assess the validity of our approach.

Multiple Compartment Model to Calculate Tyrosine Compartments in Heart Ventricles of Rats

The foregoing data suggest that a multiple compartment of heart tyrosine may be in order to explain the discrepancy found in the values of k_2 for NE efflux from heart ventricles calculated from the changes of tyrosine and NE

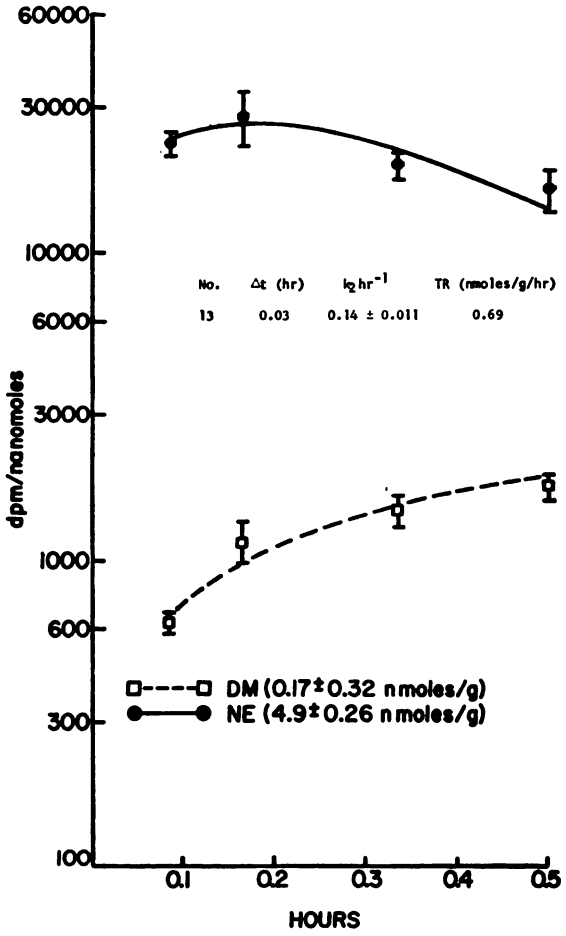


FIG. 4. Time course of the specific activity of DM and NE in heart ventricle of rats receiving 1.5 mCi/kg intravenously of 3,5-^{3}H-tyrosine. In the figure, the average k_2 calculated (according to equation X) for 13 successive 0.03-hr intervals and the turnover rate (TR)' are also shown.

specific radioactivity after pulse labeling with ^{3}H-tyrosine. The data reported in figure 4 suggest that the single NE compartmentation as indicated in the simplified model *a* is consistent not only with the estimations of NE turnover according to three of the procedures listed in table 2 but also with pulse labeling with ^{3}H-tyrosine, if the compartmentation of tissue tyrosine is by-passed. Thus, the view that NE is stored in a single compartment which kinetically behaves as if it were open at both ends is not challenged by this novel kinetic approach. Without worrying about complications and before ruling out the possibility that the answer was simple, we limited the possibility of a multiple compartmentation to the tissue tyrosine. Shown below are the basic assumptions which were followed:

1. The various compartments of tyrosine are not interconnected but they are all related to the specific activity of blood tyrosine.

2. At a time very close to "zero" (the time of the pulse injection radioactive tyrosine), the specific activity of each compartment reaches its maximum.

3. The maximal value of the specific radioactivity (S) of each compartment is proportional to its own turnover rate (k).

$$S_{TY1}(0) : S_{TY2}(0) : \cdots S_{TYn}(0) = k_{TY1} : k_{TY2} : \cdots k_{TYn}$$

To apply the multiple compartment model to the rat heart ventricle experiment, we must first consider the relationship between changes in specific radioactivity of tyrosine and DM. The data reported in figure 5 show that the specific radioactivity of heart ventricle DM is rising when the specific activity of tissue tyro-

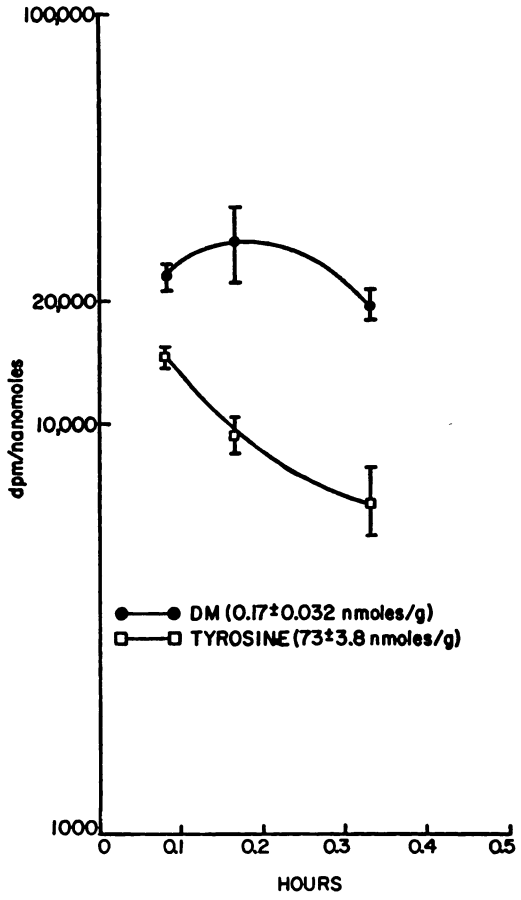


FIG. 5. Time course of the specific activity of DM and tyrosine in heart ventricles of rats receiving 1.5 mCi/kg intravenously of 3,5-^{3}H-tyrosine. Please note that the specific activity of DM reaches its maximal value at 0.166 hr while the specific activity of tyrosine is declining suggesting that this total tyrosine pool cannot be the precursors for tissue DM.

sine is declining. At all times the latter is lower than the specific radioactivity of heart ventricle DM. According to Zilversmit (41) a prerequisite compatible with a precursor product relationship is that the specific radioactivity of the precursor crosses that of the product at its highest point. The data of figure 5 certainly do not fulfill this requirement and, therefore, prevent us from considering the possibility that, from these data, we can calculate the rate constant for the efflux and the turnover rate of DM. The turnover rate of this amine measures that of NE for, as shown in model *b*, DM is the immediate and obligatory precursor of NE.

From simple inspection of the data reported in figure 5, we can infer thet the compartment of tyrosine which forms the DM has an initial specific activity greater than that of the tyrosine reported in figure 5. Moreover, if this compartment has acquired promptly a higher specific activity, it must be turning over at a rate faster than the decline of the specific radioactivity of tissue tyrosine shown in figure 5.

Let A be the total size of the multiple compartment system describing tyrosine in rat heart ventricles and A_n the relative size of the compartment n then

$$A = \sum_n A_n$$

Indicating with * the quantities of radioactive material found in each compartment, the specific activity of nth compartment is $S_n = A_n^*/A_n$. The average specific activity of the multiple compartment system is

$$S_A = \frac{1}{A} \sum_n A_n S_n \qquad \text{XV}$$

considering the case of tyrosine (TY) pertaining to our experiments (n = 1, 2) and assuming that the pulse injection of ^{3}H-tyrosine has brought instantaneously the S_{TY1} and S_{TY2} to their maximal values which decline exponentially thereafter

$$\begin{aligned} S_{TY1}(t) &= S_{TY1}(0)\, e^{-k_{TY1}t} \\ S_{TY2}(t) &= S_{TY2}(0)\, e^{-k_{TY2}t} \end{aligned} \qquad \text{XVI}$$

If we call

$$\begin{aligned} \frac{A_1^*(0)}{A_2^*(0)} &= P \\ \frac{A_1}{A_2} &= Q \end{aligned} \qquad \text{XVII}$$

then Q indicates the ratio of compartment sizes and

$$\frac{P}{Q} = \frac{S_{TY1}(0)}{S_{TY2}(0)}$$

the ratio of initial specific activity of the compartments.

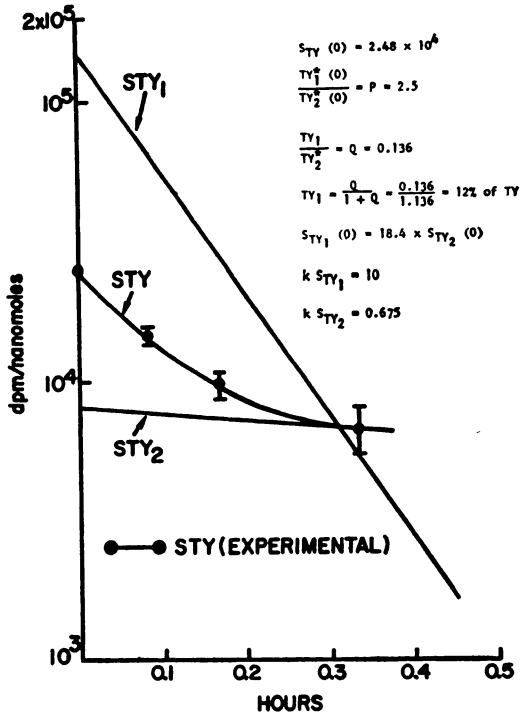


FIG. 6. Kinetic constants and compartment characteristics of tyrosine in heart ventricles of rats receiving 1.5 mCi intravenously of 3,5-^{3}H-tyrosine.

In order to solve the three unknowns—P, Q, and k_{TY1}—we use equations XXV, XXVII and XXVIII described in the appendix. The detailed mathematical derivation of these equations is given in the appendix section of this paper

$$\frac{Q}{P} = e^{k_{TY1}t_1\left(\frac{Q}{P}-1\right)} \qquad \text{XXV}$$

$$S_{TY}(t_2) = \frac{Q\,S_m\,e^{k_{TY1}t_m}}{P\,(1+Q)}\left[P\,e^{-k_{TY1}t_2} + e^{-(Q/P)k_{TY1}t_2}\right] \qquad \text{XXVII}$$

$$S_{TY}(t_3) = \frac{Q\,S_m\,e^{k_{TY1}t_m}}{P\,(1+Q)}\left[P\,e^{-k_{TY1}t_3} + e^{-(Q/P)k_{TY1}t_3}\right] \qquad \text{XXVIII}$$

substituting the following experimental values into above equations.

$$t_1 = 0.31 \text{ hr}$$

$$t_2 = 0.83 \text{ hr}$$

$$t_3 = 0.166 \text{ hr}$$

$$t_m = 0.166 \text{ hr}$$

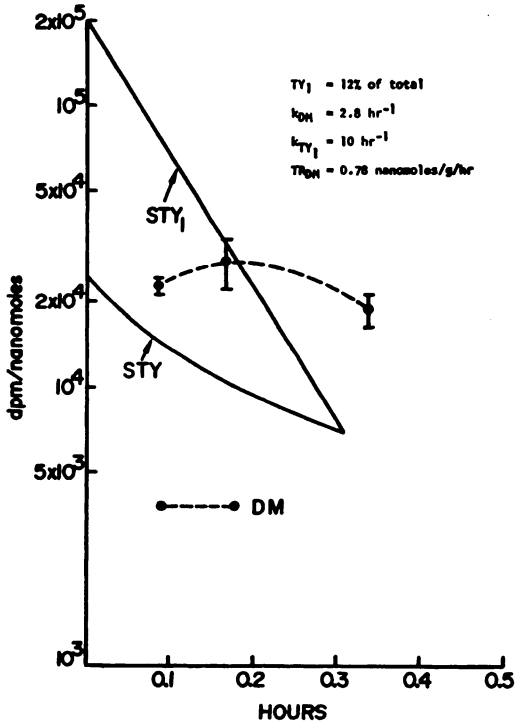


FIG. 7. Calculation of the fractional rate constant for the efflux of DM (k_{DM} or as in model B, k_1''') and DM turnover rate from the conversion of radioactive tyrosine into DM; TY1 is the tyrosine pool which is the precursor of DM and NE in rat heart ventricles.

$$S_{TY}(t_2) = 1460 \text{ dpm/moles}$$

$$S_{TY}(t_3) = 260 \text{ dpm/moles}$$

$$S_m = 28{,}000 \text{ dpm/moles}$$

we obtain (fig. 6)

$$k_{TY1} = 10$$

$$P = 2.5$$

$$Q = 0.136$$

and knowing $S_{TY1}(t)$ and the $S_{DM}(t)$ we get (k_1''' of model *b*) $k_{DM} = 2.8$ hr^{-1} as shown in figure 7. With $k_{DM} = 2.8$, and knowing that DM concentrations in rat heart ventricle are 0.29 nanomoles/g, the turnover rate of the DM pool can be estimated as 0.78 nanomoles/g/hr. This value is very close to that reported in figure 5 for turnover rate of NE calculated from the relationship between SA of DM and NE which was equal to 0.69 nanomoles/g/hr. Since it seems reasonable to assume that the synthesis of NE cannot exceed the turnover

rate of its immediate obligatory precursor, DM, we postulated that no more than 0.78 nanomoles of NE can be synthesized in 1 hr in the noradrenergic nerves present in rat heart ventricles.

Summary

The results reported have shown that mass fragmentography allows measurement of the DM pool in noradrenergic axons. By combining mass fragmentography and radiochemistry, it is possible to measure turnover rate of NE in heart ventricles from the precursor product relationship of DM and NE stored in the noradrenergic nerves. The turnover rate value obtained is compatible with those obtained by other approaches. Since the DM pool is the obligatory precursor for the NE, these data fail to give support to the view that there are several pools of NE in noradrenergic nerves which can be distinguished by virtue of their kinetic parameters.

A compartmentation can be distinguished concerning tyrosine in rat heart ventricles. From the relationship between tyrosine and DM we have calculated the size of the kinetic pool of tyrosine which serves as a precursor for DM and found that it is 12% of the total tyrosine in rat heart ventricles. Moreover, the specific radioactivity of this pool could be calculated to be 18-fold that of the rest of the tyrosine pool. These calculations of the tyrosine compartmentation rest on a number of assumptions which include the postulate that the DOPA pool is very small and that the transformation from DOPA to DM is practially instantaneous. The conversion by a soluble enzyme of DOPA into DM occurring in the cytoplasm supports this possibility.

The formation of DM, from a tyrosine pool, kept in a compartment which we have defined only kinetically yields a k_1''' (model *b*) of 2.8 hr^{-1} indicating that 0.78 nanomoles/g/hr of DM turnover in 1 hr. By measuring the precursor product relationship between NE and DM we have obtained a turnover of NE of 0.69 nanomoles/g/hr. It is concluded that this value represents a reliable approximation of the absolute rate of NE synthesis in heart ventricles of rats.

Appendix

The mathematical derivation of the equations for the two compartments (TY1 and TY2) is detailed as follows.

After the pulse injection, we have assumed that the specific activity of both compartments rises very rapidly to its maximal values and declines exponentially thereafter, *i.e.*:

$$S_{TY1}(t) = S_{TY1}(0)e^{-k_{TY1}t}$$
$$S_{TY2}(t) = S_{TY2}(0)e^{-k_{TY2}t} \qquad \text{XVI}$$

We have further assumed that these relative maximal values are in direct proportion to their turnover rate. This assumption together with the definition

$$\frac{A_1^*(0)}{A_2^*(0)} = P$$
$$\frac{A_1}{A_2} = Q \qquad \text{XVII}$$

TABLE 7

Theoretical possibilities for the relationships of various parameters in a double compartment

Possibility	P	Q	k_{TY1}	k_{TY2}	k_{TY}
I	3.0	1.0	5.0	1.66	3.54
II	5.0	1.0	5.0	1.0	3.58
III	5.0	0.5	5.0	0.5	3.27

k_{TY} , composite fractional rate constant approximated by simple exponential decay.

We can then express k_{TY2} in terms of k_{TY1} by the relationship below

$$k_{TY2} = \frac{k_{TY2}}{k_{TY1}} k_{TY1} = \frac{S_{TY2}(0)}{S_{TY1}(0)} k_{TY1} = \frac{Q}{P} k_{TY1} \qquad \text{XVIII}$$

The average specific activity of TY is called S_{TY}

$$S_{TY} = \frac{Q}{1+Q} S_{TY1} + \frac{1}{1+Q} S_{TY2} \qquad \text{XIX}$$

or in a normalized form

$$\frac{S_{TY}}{S_{TY2}(0)} = \frac{1}{1+Q} [P\, e^{-k_{TY1}t} + e^{-(Q/P)k_{TY1}t}] \qquad \text{XX}$$

The complications entailed in a two compartment system are illustrated in table 7. These data show that an apparently similar composite value of k (k equ) can be obtained with large variations of the other k values. Table 7 gives an appreciation of the problems involved in these evaluations, and shows the uncertainties concealed in the mathematics of multiple compartments.

Since $S_{TY1}(t)$ has to interact with the $S_{DM}(t)$ curve at the maximal point of the latter we can write that

$$S_{TY1}(t_m) = S_{TY1}(0)e^{-k_{TY1}t_m} = S_m \qquad \text{XXI}$$

where t_m is the time when $S_{DM}(t)$ reaches its maximal value and S_m is that maximal value. Now we also observe (fig. 6) that the $S_{TY1}(t)$ curve and $S_{TY2}(t)$ curve and the $S_{TY}(t)$ have to intersect at a single point at time t_1 , *i.e.*

$$S_{TY2}(t_1) = S_{TY1}(t_1) \qquad \text{XXII}$$

Since

$$S_{TY2}(t_1) = S_{TY2}(0)\, e^{-k_{TY2}t_1} = \frac{Q}{P} S_{TY1}(0)\, e^{-(Q/P)k_{TY1}t_1} \qquad \text{XXIII}$$

and

$$S_{TY1}(t_1) = S_{TY1}(0)e^{-k_{TY1}t_1}\,. \qquad \text{XXIV}$$

Substitute XXIII and XXIV into XXII, we obtain the relationship

$$\frac{Q}{P} = e^{k_{TY1}t_1}\left(\frac{Q}{P} - 1\right) \qquad \text{XXV}$$

If we now substitute XVIII and XXI into equation XX in order to eliminate the unknown, $S_{TY_2}(0)$, we can rewrite equation XX in the form

$$S_{TY}(t) = \frac{QS_m e^{-k_{TY_1}t_m}}{P(1+Q)} [P e^{-k_{TY_1}t} + e^{-(Q/P)k_{TY_1}t}] \qquad \text{XXVI}$$

In order to solve the three unknowns—P, Q, and k_{TY_1}—we use equation XXV and the following two equations obtained from using experimental data of $S_{TY}(t)$ with equation XXVI at two data points, t_2 and t_3, *i.e.*,

$$S_{TY}(t_2) = \frac{QS_m e^{-k_{TY_1}t_m}}{P(1+Q)} [P e^{-k_{TY_1}t_2} + e^{-(Q/P)k_{TY_1}t_2}] \qquad \text{XXVII}$$

and

$$S_{TY}(t_3) = \frac{QS_m e^{-k_{TY_1}t_m}}{P(1+Q)} [P e^{-k_{TY_1}t_3} + e^{-(Q/P)k_{TY_1}t_3}] \qquad \text{XXVIII}$$

where $S_{TY}(t_2)$, $S_{TY}(t_3)$ should be chosen at points with minimal uncertainties. Therefore, equations XXV, XXVII and XXVIII together will give unique solutions for P, Q and k_{TY_1}.

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