PRIMARY CULTURES OF DISSOCIATED SYMPATHETIC NEURONS

II. Initial Studies on Catecholamine Metabolism

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ABSTRACT

Initial studies are reported on the catecholamine metabolism of low-density cultures of dissociated primary sympathetic neurons. Radioactive tyrosine was used to study the synthesis and breakdown of catecholamines in the cultures. The dependence of catecholamine synthesis and accumulation on external tyrosine concentration was examined and a concentration which is near saturation, 30 μM, was chosen for further studies. The free tyrosine pool in the nerve cells equilibrated with extracellular tyrosine within 1 h; the total accumulation of tyrosine (free tyrosine plus protein, catecholamines, and metabolites) was linear for more than 24 h of incubation. Addition of biopterin, the cofactor of tyrosine hydroxylase, only slightly enhanced catecholamine biosynthesis by the cultured neurons. However, addition of reduced ascorbic acid, the cosubstrate for dopamine β-hydroxylase, markedly stimulated the conversion of dopamine (DA) to norepinephrine (NE). Phenylalanine, like tyrosine, served as a precursor for some of the DA and NE produced by the cultures, but tyrosine always accounted for more than 90% of the catecholamines produced.

The DA pool labeled rapidly to a saturation level characteristic of the age of the culture. The NE pool filled more slowly and was much larger than the DA pool. The disappearance of radioactive NE and DA during chase experiments followed a simple exponential curve. Older cultures showed both more rapid production and more rapid turnover of the catecholamines than did younger cultures, suggesting a process of maturation.

INTRODUCTION

The initial paper in this series (Mains and Patterson, 1973 *a*) described the conditions for the isolation and growth of sympathetic neurons in low-density cultures and described some of the basic morphological and biochemical properties of the neurons. It is hoped that this culture system will prove useful in the study of neurospecificity and neuronal development. Toward this end we have begun a study of catecholamine (CA)[1] metabolism in the cultures in order to characterize further the neurons as well as to obtain information on

[1] *Abbreviations used in this series of papers:* ACh, acetylcholine; BH_4, tetrahydrobiopterin; BSA, bovine serum albumin; CA, catecholamine; DA, dopamine,

certain basic questions of catecholamine biochemistry not easily approached in vivo.

The pathway of biosynthesis of the catecholamines from tyrosine involves three enzymes and several cosubstrates and cofactors (for a recent review see Molinoff and Axelrod, 1971). Tyrosine hydroxylase (TH) converts tyrosine plus a reduced pteridine to 3,4-dihydroxyphenylalanine (dopa) plus an oxidized pteridine. Dopa is converted by aromatic amino acid decarboxylase to 3,4-dihydroxyphenethylamine (dopamine; DA). Finally, dopamine β-hydroxylase (DBH) oxidizes reduced ascorbate and hydroxylates DA to form 1-(3,4-dihydroxyphenyl)-2-aminoethanol (norepinephrine; NE). Molecular oxygen is required for both the first and third reactions.

The preceding paper presented data showing that the cultured sympathetic neurons synthesize and accumulate radioactive DA and NE when incubated in radioactive tyrosine (Mains and Patterson, 1973 *a*). Here we present studies on nutritional requirements for catecholamine synthesis, as well as kinetic analyses of NE and DA biosynthesis and disappearance.

MATERIALS AND METHODS

Much of the methodology used in the experiments described here is the same as was described in the preceding paper (Mains and Patterson, 1973 *a*). Except where noted, preparation and maintenance of the cultures, radioactive compounds used, and methods of incubation were the same. Cultures were grown and incubated with 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine ($DMPH_4$) (0.5 mg/l; Aldrich Chemical Co., Inc., Milwaukee, Wis.); fresh ascorbic acid (25 mg/liter) was added every 2 h during incubations, except where stated. L-[7-^{3}H]norepinephrine (6.4 Ci/mmol; New England Nuclear, Boston, Mass.) and L-[3-^{3}H]phenylalanine (16 Ci/mmol; New England Nuclear) were also used. Phenylalanine was purified by paper electrophoresis as described previously. The [3,5-^{3}H]tyrosine was always 30 Ci/mmol (New England Nuclear).

3,4-dihydroxyphenethylamine; DBH, dopamine β-hydroxylase; $DMPH_4$, 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine; dopa, 3,4-dihydroxyphenylalanine; FCS, fetal calf serum; GABA, γ-aminobutyric acid; 5-HT, 5-hydroxytryptamine (serotonin); L-15, Leibovitz's medium; MEM, Eagle's minimal essential medium; αMPT, α-methyl-p-tyrosine; NE, norepinephrine (1-[3,4-dihydroxyphenyl]-2-aminoethanol); NGF, nerve growth factor; PCA, perchloric acid; SCG, superior cervical ganglion; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; TH, tyrosine hydroxylase.

Tyrosine Concentration Dependence

The concentration of tyrosine was varied in the incubation medium by the addition of appropriate aliquots of Leibovitz's (L-15-air) plating medium. Rat serum contributed 3 μmol/liter unlabeled free tyrosine to the final incubation mix, since rat serum contains about 75 μM tyrosine (Mains and Patterson, 1973 *a*).

Ascorbic Acid Determinations

The methods of Baker and Frank (1968) and Roe (1954) were used to determine total ascorbate (reduced plus oxidized plus diketogulonic acid) and reduced ascorbate, respectively.

Phenylalanine Incubations

To compare phenylalanine and tyrosine as precursors for catecholamine synthesis, cultures were incubated with [^{14}C]tyrosine (455 mCi/mmol) and [^{3}H]phenylalanine. The tyrosine concentration was always 30 μM; phenylalanine was varied from 30 to 750 μM. Concentrations of 30–250 μM were tested using [^{3}H]phenylalanine (16 Ci/mmol). For economy and to make the contributions of each precursor more easily separable, 750 μM phenylalanine was tested using identical cultures incubated with 30 μM [^{3}H]-tyrosine plus 750 μM unlabeled L-phenylalanine, or 30 μM unlabeled tyrosine and 750 μM [^{3}H]L-phenylalanine (4.25 Ci/mmol). Catecholamines formed from [^{3}H]phenylalanine were determined by electrophoresis followed by chromatography, as previously described. Since all evidence in vivo (Long, 1961; Meister, 1965) and in vitro (Eagle, 1955 and 1959) indicates that mammalian cells do not utilize D-amino acids, nor do D-amino acids compete with L-amino acids even in ten-fold excess, we have ignored the presence of D-phenylalanine in L-15.

Incubations with Radioactive Norepinephrine

Cultures were pulsed with 0.25 μM [^{3}H]norepinephrine for 1 h; then the chase procedure was followed.

Chase Experiments

Chase experiments were performed on cultures incubated for various times in [^{3}H]tyrosine, or for 1 h in [^{3}H]NE. The cultures were washed after the incubation with 2 ml of L-15-air without Methocel, drained and then incubated in another 2 ml of L-15-air growth medium without Methocel, giving at least a 1000× dilution of residual [^{3}H]NE left in the dish. Fresh ascorbic acid (25 mg/liter) was supplied every 2–3 h during the chase.

RESULTS

Dependence of Catecholamine Production on External Tyrosine Concentration

Since neurons utilize tyrosine for several purposes, studies of catecholamine metabolism could give misleading results if the concentration of tyrosine were low enough to be limiting, thereby forcing incorporation of tyrosine into protein to compete with conversion into neurotransmitter. In these cultures an assessment of the routes of tyrosine utilization can be attempted. Cultures aged 1, 2, and 3 wk were incubated in varying concentrations of tyrosine for 8-h periods and several parameters were measured. Fig. 1 presents the pooled results from experiments on the cultures. In Fig. 1 A, it can be seen that the free radioactive tyrosine pool of the cultures expands with increases in external radioactive tyrosine concentration at least to 200 μM tyrosine. Although the free tyrosine pool expands with increasing extracellular tyrosine, the total radioactivity accumulated by the cells (largely present as protein, since it comigrates with the major protein bands seen on stained sodium dodecyl sulfate (SDS) polyacrylamide gels of the cultures; unpublished observations) reaches a plateau between 30 and 60 μM external tyrosine as shown in Fig. 1 B. These values are to be compared with 75 μM, the value for rat serum (Mains and Patterson, 1973 *a*). [^{3}H]catecholamine synthesis and accumulation from extracellular [^{3}H]tyrosine also reaches a plateau near the physiological range of tyrosine values, as seen in Fig. 1 C. For subsequent work, 30 μM tyrosine has been used.

Time Dependence of Uptake and Accumulation of Exogenous Tyrosine

An understanding of catecholamine metabolism in these cultures requires data concerning the time dependence of tyrosine uptake and accumulation, as well as the temporal patterns of the production of DA and NE from tyrosine. As a first step in this study, it was important to determine if the extracellular tyrosine equilibrated rapidly with the neuronal tyrosine pool. This seemed likely, since the free amino acid pools of various non-neural cells in culture are known to exchange with the surrounding medium in less than an hour (Eagle, 1959). Therefore, a series of cultures were incubated in [^{14}C]tyrosine for 8 h and then an aliquot of [^{3}H]tyrosine was added to the medium;

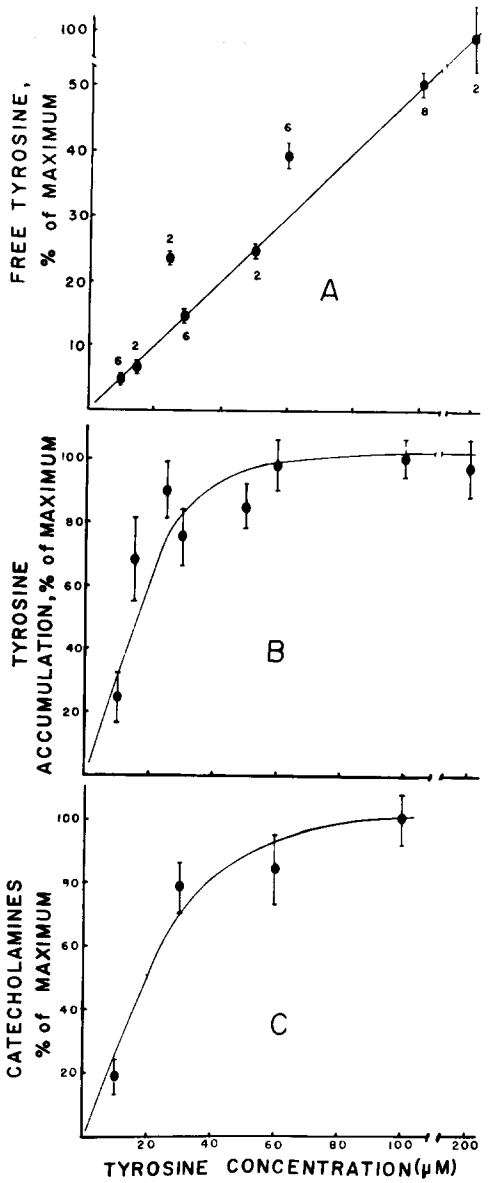

FIGURE 1 1-, 2-, and 3-wk old cultures were incubated for 8 h with radioactive tyrosine at various concentrations (as indicated). The radioactivity in free tyrosine (A), total accumulation (B), and in catecholamine (C) was determined as described in Materials and Methods. The data from cultures of three ages were pooled and the small numbers near the bars are the number of cultures analyzed at each concentration. The bars represent the SEM (in all figures).

the cpm ratio ^{3}H/^{14}C in the medium was 9.8. The ^{3}H/^{14}C ratio of the free tyrosine pool was then monitored as a function of time. Within 1 h, the ratio ^{3}H/^{14}C in the intracellular pool was 9.9 ±

0.2, the same as the extracellular medium. In nine experiments with sets of identical cultures in which only [^{3}H]tyrosine was used in the medium, the free [^{3}H]tyrosine peak rose abruptly to its final value at the earliest time point, and remained at that value for periods up to 28 h of incubation (e.g., Fig. 2, open circles).

The rapid approach to a steady state of the intracellular free tyrosine need not represent bidirectional exchange, since the cells utilize an amount of tyrosine greater than the size of their free tyrosine pool every hour (see below).

To interpret the tyrosine concentration dependence shown in Fig. 1, it is necessary to determine whether the data represent the rate or extent of synthesis and accumulation. Two sets of 1-wk old cultures were used to examine the kinetics of labeling, and the pooled data are shown in Fig. 2. Cultures were incubated for varying lengths of time and then harvested and analyzed. As seen in Fig. 2, the total accumulation of radioactivity was linear with incubation time. Thus the 8-h incubation data in Fig. 1 B represent rates, not steady state values. Also, in seven experiments using older (2-wk) cultures, total accumulation of radioactivity was found to be linear with incubation time (e.g., Fig. 5 A). Thus the rapid exchange of free tyrosine and the linear accumulation of total radioactivity occur in both young and older cultures. Since there is little or no proliferation of non-neural cells in these cultures and the neurons apparently do not divide (Mains and Patterson, 1973 *a*), the steady accumulation of radioactivity with time represents growth and turnover in the neurons.

Phenylalanine as Precursor for Catecholamine Biosynthesis

It has been reported that phenylalanine as well as tyrosine can serve as precursor for catecholamine synthesis in brain homogenates (Karobath and Baldessarini, 1972). Tyrosine hydroxylase is inhibited by tyrosine but not by phenylalanine, and the isolated enzyme can produce dopa by doubly hydroxylating phenylalanine faster than by single hydroxylation of tyrosine (Shiman et al., 1971). The question remains, however, whether phenylalanine can serve as a catecholamine precursor in intact cells. If so, it is important to know how much of the catecholamine synthesized and accumulated comes from tyrosine and how much from phenylalanine.

To study the two potential precursors, cultures were incubated with both [^{14}C]tyrosine and [^{3}H]-phenylalanine for 8 h, and DA and NE were isolated by paper electrophoresis and chromatography. The results of a series of experiments appear in Fig. 3, in which the fraction of catecholamines synthesized from tyrosine (relative to the total synthesis from both phenylalanine and tyrosine) is plotted as a function of external phenylalanine concentration (all at 30 μM tyrosine). Phenylalanine served as precursor for both DA and NE, and competed with tyrosine as precursor in a graded manner. However, even at the highest concentration of phenylalanine tested, 750 μM, phenylalanine accounted for less than 10% of the catecholamines synthesized, in spite of the fact that the cultures took up and accumulated almost eight times more phenylalanine than tyrosine. Furthermore, phenylalanine accounted for less than 10% of the catecholamines synthesized even when the tyrosine concentration was 10 μM. Deletion of $DMPH_4$ from growth and incubation media did not significantly alter the results of the mixed tyrosine-phenylalanine experiments.

Effects of Exogenous Pteridine Cofactor and Exogenous NE

We investigated the possibility that catecholamine metabolism is regulated by the supply of biopterin, the pteridine cofactor for tyrosine hydroxylase, by assaying for a stimulation of catecholamine production upon the addition of exogenous biopterin (BH_4), or its synthetic analog $DMPH_4$. (The biopterin was the generous gift of Dr. Seymour Kaufman). Exps. A, B, and C of Table I show that, although there may be a small stimulation using fresh BH_4 or $DMPH_4$ during incubations with radioactive tyrosine, the effects were not statistically significant. The cultures were grown in L-15 lacking folic acid and fresh vitamin mix; the medium was supplemented at every feeding with fresh ascorbic and folic acids (50 and 1 mg/liter, respectively). This was done in an effort to minimize spontaneous formation in the medium of biologically active derivatives of folic acid which can substitute for biopterin (Lloyd et al., 1971). We hoped to force the cells either to synthesize biopterin, acquire biopterin from the serum, or to survive with a deficit of the pteridine cofactor.

Another approach to the study of pteridine cofactors involved the use of exogenous unlabeled

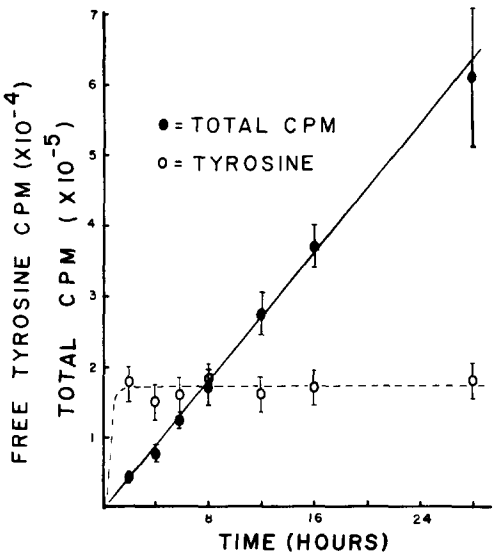

FIGURE 2 8-day old cultures were incubated for varying times (as indicated) with 30 μM radioactive tyrosine. The open circles represent the radioactivity in free tyrosine and the closed circles give the total radioactivity accumulated at each time point.

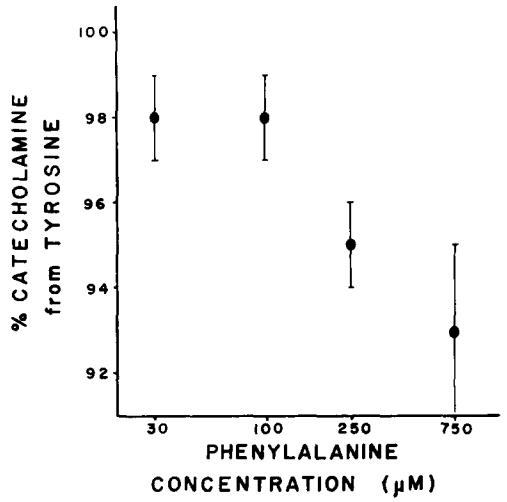

FIGURE 3 3–4-wk old cultures were incubated with various concentrations of [^{3}H]phenylalanine and 30 μM [^{14}C]tyrosine for 8 h as described in Materials and Methods. The amounts of [^{3}H]- and [^{14}C]catecholamines were then determined. Each point is the mean of triplicate determinations.

NE to inhibit the production of new catecholamines from radioactive tyrosine. Table II shows that exogenous NE inhibited the production of radioactive catecholamines, and that the effect of exogenous NE was stronger on NE than on DA synthesis and accumulation. Since NE competes with pteridine cofactors in binding to tyrosine hydroxylase (Nagtasu et al., 1972), it was possible that BH_4 or $DMPH_4$ would relieve blockade of [^{3}H]-catecholamine synthesis and accumulation produced by exogenous unlabeled NE. Exps. C and D of Table I indicate that added cofactor had no significant effect on NE inhibition, although a further small inhibition of catecholamine production may have been caused by the pteridines.

Effects of Exogenous Ascorbic Acid

Early in these studies we observed that as the cultured nerve cells grew for progressively longer times in culture, they synthesized and accumulated more DA than NE. For 8-h incubations, the ratio NE/DA in older cultures was often as low as 0.1. This was unexpected since the cultured neurons appeared morphologically to be principal cells, which in vivo produce primarily NE, not DA, and since the growth and incubation media always contained ascorbic acid, the cosubstrate for dopamine-β-hydroxylase. Upon examination, it became clear that ascorbic acid is extremely labile under culture conditions, and must be frequently supplied fresh in the culture medium if the neurons are to synthesize and store substantial amounts of NE. As seen in Table III A, cultures grown for 7 days in 50 mg/liter ascorbic acid supplied fresh daily produce 2.5 times as much radioactive NE in an 8-h period as cultures grown for 7 days without exogenous ascorbate; no significant differences in DA production or total tyrosine accumulation are seen. In Table III B are presented the results of growing cultures for 2 wk without fresh ascorbate and then adding ascorbate for the 16-h incubation; NE synthesis and accumulation was stimulated tenfold, while the DA pool was actually made smaller by the addition of ascorbate.

Although ascorbic acid is unstable, it was possible that the neurons stored ascorbate for subsequent use. To examine this question, cultures were grown for 3 wk with fresh ascorbate daily and then incubated for 8 h with radioactive tyrosine plus either fresh ascorbate or an equal aliquot of incubation medium which had been preincubated in the absence of cells at pH 7.2, 36°C, for 16 h (Table III C). The synthesis and accumulation of NE was stimulated over sixfold by fresh ascorbate, again with no change in the DA pool or the total tyrosine accumulated. Thus the neurons must be supplied with ascorbate more

TABLE I

Effects of Exogenous NE and Exogenous Pteridine Cofactors on CA Synthesis and Accumulation

Exp.	Exogenous pteridine		Exogenous	Total	NE	DA	CA
	BH_4	$DMPH_4$	NE				
	mg/liter		*μM*	*cpm* × 10^{-3}	*cpm*	*cpm*	*cpm*
A	0	0	0	195	2,500	1,200	3,700 ± 700
	0	0.5	0	180	3,700	1,600	5,300 ± 700
B	0	0	0	305	8,400	4,800	13,200 ± 2,000
	0	0.5	0	315	13,000	6,300	19,300 ± 3,000
C	0	0	0	190	5,300	2,100	7,400 ± 1,200
	5	0	0	175	8,600	2,900	11,500 ± 1,600
	0	0	2	155	500	300	800 ± 100
	5	0	2	185	250	250	500 ± 130
D	0	0.5	0	210	8,300	6,100	14,400 ± 1,700
	0	0.5	3	200	1,050	1,000	2,050 ± 700
	0	5.0	3	210	300	600	900 ± 300

All data presented as the mean of triplicate determinations; all incubations were for 8 h in 30 μM [^{3}H]tyrosine; SEM was within ±20% in all cases for which error bounds are not shown. Total cpm = the total radioactivity accumulated in a culture in 8 h.

Exp. A: Grown 8 days in folate-minus medium which was supplemented at every feeding with 1 mg/liter fresh folate; grown and incubated in presence or absence of $DMPH_4$, as indicated.

Exp. B: Same as exp. A, except 15 days old.

Exp. C: Grown 14 days without exogenous pteridine; various levels of NE and BH_4 introduced during incubation.

Exp. D: Grown 14 days in 0.5 mg/liter $DMPH_4$; various levels of $DMPH_4$ and NE introduced during the incubation.

often than once daily if they are to synthesize their full complement of NE. Other experiments showed that added glutathione (50 mg/liter) had no effect on the conversion of DA to NE and thus would not substitute for ascorbate.

Peterkofsky (1972 *a*) showed that total ascorbic acid (reduced plus oxidized ascorbate plus diketogulonic acid) in Eagle's minimal essential medium (MEM) decays to very low levels in 16 h when incubated at 37°C, pH 7.4; we have confirmed these results using L-15-air. Dopamine β-hydroxylase requires reduced ascorbate to convert DA to NE (Levin and Kaufman, 1961); once it becomes oxidized, ascorbate is converted to diketogulonate in a matter of minutes under tissue culture conditions (Ball, 1937). Since ascorbate is made primarily in the liver and kidney, it is possible that nerve cells cannot resynthesize reduced ascorbate from diketogulonic acid (Pauling, 1970; Baker and Frank, 1968). Therefore we measured the loss of reduced ascorbic acid from L-15 at a variety of pH values, with and without serum, and with and without added glutathione (50 mg/liter). In all cases, less than 1% of the initial reduced ascorbate (50 mg/liter) was present after 2 h of incubation, even when special precautions (lower pH, inclusion of reduced glutathione) were taken to stabilize the ascorbate.

Because ascorbic acid is so unstable, it was supplied fresh every 2–3 h in all subsequent incubations.

Time Dependence of Catecholamine Production from Exogenous Tyrosine

The kinetics of NE and DA metabolism were more complicated than in the case of the free tyrosine and total tyrosine discussed above. In 1-wk old cultures treated daily with ascorbic acid (Fig. 4) and supplied with ascorbate every 2–3 h during the incubation as well, the DA pool labeled maximally in 2–4 h and then did not change for the next 28 h. NE, on the other hand, labeled more gradually and in fact showed no sign of saturation

for at least 28 h of incubation. The same pattern was observed when cultures were not grown in ascorbate, though ascorbate addition during the incubation was essential for maximal NE synthesis and accumulation (Table III).

TABLE II

Inhibition of Catecholamine Synthesis and Accumulation by Exogenous Norepinephrine

Exogenous NE	Total	NE	DA	CA	NE/DA
μM	*cpm* × 10^{-3}	*cpm*	*cpm*	*cpm*	
0	290	4,700	5,000	9,700	1.0
0.1	270	4,000	4,900	8,900	0.8
0.5	310	4,900	4,900	9,800	1.0
1.0	240	1,225	3,000	4,225	0.3
10.	265	50	100	150	*

12-day old cultures were incubated for 8 h in 30 μM [^{3}H]tyrosine in the presence of various concentrations of NE. Ascorbic acid (25 mg/liter) was supplied fresh every 2 h during the incubation. The cultures were grown and incubated without added pteridine cofactor. Data is the mean of quadruplicate determinations; SEM was always ±15% or less.

* = 0.5 ± 0.4; number is not significant, because DA and NE cpm were very low.

Total cpm = the total radioactivity accumulated by the culture in 8 h.

In 2-wk old cultures, the rate of catecholamine production was greatly accelerated compared to the younger cultures (Fig. 4), as shown by the open circles for NE in Fig. 5 B.

The effect of ascorbate on the time course of NE labeling is also shown in this figure. Cultures were either maintained without ascorbate until the incubation (open circles) or were given fresh ascorbate (25 mg/liter) plus glutathione (15 mg/liter) every 12 h for 4 days before the incubation (closed circles). It was thought that these feeding protocols would produce cultures with small and large endogenous NE pools, respectively, so that the effects of the endogenous NE level on the kinetics of labeling could be observed. All cultures were given ascorbate and glutathione every 2–3 h during the incubations. The total tyrosine incorporation by the two sets of cultures was identical for 16 h (Fig. 5 A), indicating that ascorbate prefeeding did not affect the overall tyrosine utilization. In both types of culture, the radioactivity in DA remained at 5000 ± 700 cpm for all times examined. On the other hand, the cultures grown without ascorbate produced NE at a linear rate for 16 h, while cultures maintained in ascorbic acid produced radioactive NE at a much slower rate. In two other experiments, cultures fed daily with ascorbate showed the linear, rapid NE labeling pattern, while four experiments with cultures fed two or more times daily with ascorbate

TABLE III

Effect of Ascorbic Acid on Catecholamine Biosynthesis

Exp	Age of culture	Ascorbate during growth*	Ascorbate during incub'n‡	Length of incub'n	Total	NE	DA	NE/DA
	days			*h*	*cpm* × 10^{-3}	*cpm*	*cpm*	
A	7	−	−	8	165 ± 45	1,450 ± 350	2,300 ± 450	0.6
	7	+	+	8	225 ± 50	3,700 ± 700	2,050 ± 300	1.7
						$p < 0.02$		
B	13	−	−	16	560 ± 60	1,400 ± 600	11,700 ± 3,000	0.1
	13	−	+	16	590 ± 50	13,700 ± 1,000	5,300 ± 700	2.7
						$p < 0.001$		
C	21	+	−	8	480 ± 45	2,700 ± 500	9,650 ± 1,900	0.3
	21	+	+	8	430 ± 70	17,900 ± 2,300	8,200 ± 1,700	2.3
						$p < 0.001$		

Data presented as the mean ± SEM for quadruplicate determinations. Total cpm = the total radioactivity accumulated by the culture during the incubation.

* 25 mg/liter added daily; medium changed every 3 days.

‡ 25 mg/liter added fresh every 2–3 h.

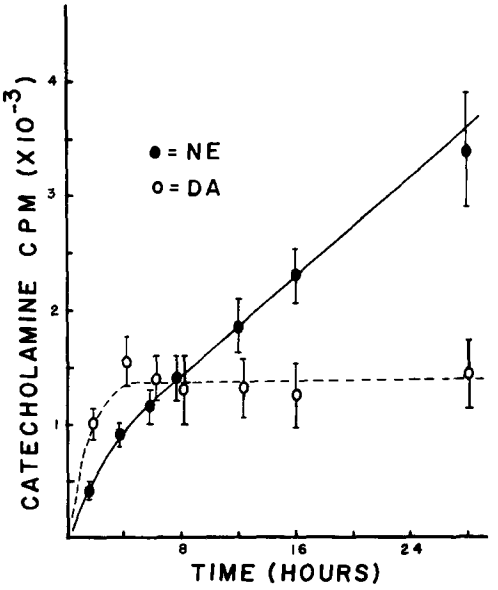

FIGURE 4 8-day old cultures were incubated for various times (as indicated) with 30 μM radioactive tyrosine. The open circles represent the radioactivity in DA, while the closed circles give the radioactivity in NE at each time. The data for each point is the mean ± SEM of 3–6 cultures. The cultures were grown with fresh ascorbic acid added daily and the incubations were in the presence of ascorbate (50 mg/ml; added fresh every 2–3 h).

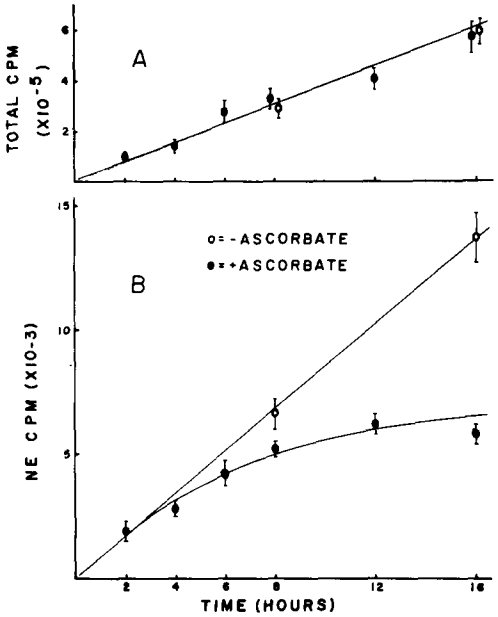

FIGURE 5 Cultures were grown for 2 wk and then incubated with radioactive tyrosine for the indicated times. One set of cultures was grown without ascorbate additions (indicated by the open circles) and one set received twice daily additions of fresh ascorbic acid for the 4 days before the incubations (indicated by the closed circles). All cultures received fresh ascorbate additions every 2–3 h during the incubations. Each point is the mean of triplicate determinations.

showed NE labeling patterns similar to the closed circles in Fig. 5 B. As already seen in Table II, NE can inhibit the production of [^{3}H]catecholamine from [^{3}H]tyrosine; endogenous stores of NE due to frequent ascorbate additions may account for the labeling patterns in Fig. 5 B.

Turnover of Catecholamines; Chase Experiments

Having obtained some information on the synthesis of catecholamines in the cultures, it was of interest to examine neurotransmitter breakdown or loss as well. After labeling a set of 1-wk old cultures for 8 h, the data from chase experiments in unlabeled tyrosine revealed simple exponential decays for both NE and DA, as seen in Fig. 6. 2-wk old cultures also exhibited exponential decline of labeled NE and DA, and these results are summarized in Table IV. An alternative method for observing NE decay, which is often used in vivo, involved a short incubation with [^{3}H]NE at a concentration (0.25 μM) that did not alter the overall rate of NE production (see Table II), followed by incubation in the absence of exogenous NE. An example of such a chase following a 1-h incubation of a set of 2-wk old cultures in 0.25 μM [^{3}H]NE is given in Fig. 7. This method also shows that the radioactive NE disappearance follows a single exponential with the same half-time as seen using radioactive tyrosine to label the NE. The data from experiments using the two methods of determining turnover rate are compared in Table IV. The table includes the results of a chase experiment done on cultures fed twice daily with fresh ascorbate for 4 days before the incubation with radioactive tyrosine; in addition, the incubation was carried out for 16 h rather than the usual 8. These manipulations were used in an effort to preload the neuronal stores with as much NE as possible. The data from the chase experiment was, however, identical to that seen previously. The chase results were not altered by maintaining cultures in the absence of penicillin-streptomycin, imidazole, or serum.

Two further considerations were examined and

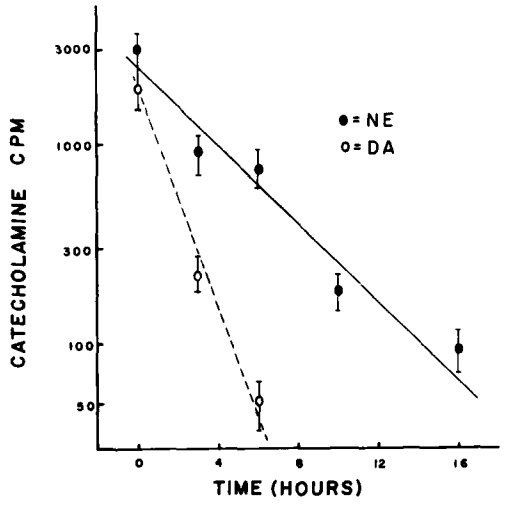

FIGURE 6 8-day old cultures were incubated 8 h with radioactive tyrosine (with ascorbate additions) and then chased in complete medium (minus Methocel) as described in Materials and Methods. The open circles refer to the radioactivity in DA and the closed circles indicate the NE radioactivity.

found not to change the rates of disappearance of radioactive CA's. The tyrosine concentration in the chase medium could have affected the rate of synthesis and thereby possibly the turnover rates. This did not seem likely however, in view of the data presented in Fig. 1 which show that the rate of catecholamine synthesis was not significantly increased at tyrosine concentrations above 30 μM. Experiments directed at this question, Table IV B, show that similar chase rates were obtained with tyrosine concentrations of 30 and 1550 μM in the chase medium. Another possibility was that merely changing the medium might affect the neurons in some way so as to give abnormal chase rates. To test this possibility, cultures were incubated for 8 h with 50 μM [^{14}C]tyrosine; then a small aliquot of medium containing [^{3}H]tyrosine (net 33 μM) was added and the incubation continued for varying times. The chase rate was calculated from the appearance of [^{3}H]catecholamines (which in a normal chase would have been unlabeled) corrected for the concomitant production of new [^{14}C]catecholamines. This experiment (Table IV, exp. 7)

TABLE IV

Chase Experiments on 1- and 2-Wk Old Cultures

Experiment	Labeling time	Longest time of chase	$t_{1/2}$ NE	$t_{1/2}$ DA
	h	*h*	*h*	
A. 7–8-day cultures				
1 (*a*, *c*)	8	16	3.2	1.3
2 (*a*, *c*)	8	6	2.3	0.95
3 (*b*, *c*)	1	9	3.3	
Mean ± SEM			2.9 ± 0.3	1.1 ± 0.2
B. 13–14-day cultures				
4 (*a*, *c*)	8	10	0.9	0.4
5 (*a*, *c*)	8	4	1.2	0.6
6 (*a*, *d*)	8	10	1.5	0.9
7 (*e*)	8	8	1.0	0.2
8 (*a*, *d*, *f*)	16	8	1.1	0.6
9 (*b*, *d*)	1	4	1.1	
Mean ± SEM			1.1 ± 0.1	0.5 ± 0.1

Chase experiments were performed as described in Materials and Methods. At least four time points, including zero time, were taken in each chase. In all experiments, data were consistent with a single straight line when plotted in semilog fashion. All time points done in triplicate. Symbols: (*a*) Incubation in 30 μM [^{3}H]tyrosine, (*b*) Incubation in 0.25 μM [^{3}H]NE, (*c*) Chase with 1550 μM tyrosine, (*d*) Chase with 30 μM tyrosine, (*e*) Incubation in [^{14}C]tyrosine, supplemented with [^{3}H]tyrosine without changing the medium; chase was calculated from rate of synthesis of new NE and DA from [^{3}H]tyrosine; for details see text. (*f*) Fed ascorbate (25 mg/liter) twice daily for the 4 days before the incubation in [^{3}H]tyrosine.

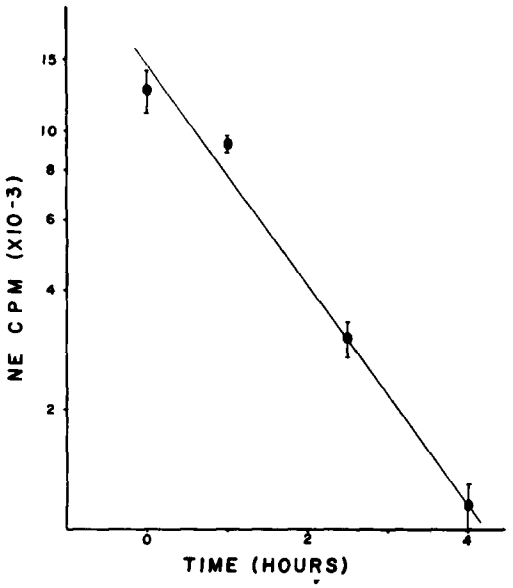

FIGURE 7 2-wk old cultures were pulsed for 60 min with [^{3}H]NE as described in Materials and Methods and then chased in 30 μM unlabeled tyrosine for the indicated times. The cultures had been given fresh ascorbate twice daily for the 4 days before the pulse as well as every 2–3 h during the chase.

showed that the chase rates obtained with a negligible change of the medium were the same as when the bulk of the medium was changed.

DISCUSSION

As part of the characterization of the neuronal cultures before the addition of other cell types, this paper has presented studies on certain nutritional and kinetic aspects of catecholamine metabolism in dissociated sympathetic neurons. Some of these findings may be relevant to questions about catecholamine metabolism raised in investigations of sympathetic neurons in vivo.

Tyrosine Metabolism: Concentration Dependence

To simplify the interpretation of the catecholamine synthesis and disappearance in the cultures, the isotopic incubations were carried out under conditions where the tyrosine concentration was not limiting; the uptake and accumulation of tyrosine was linear throughout the incubation period. The dependence of both tyrosine accumulation and catecholamine production on external tyrosine concentration shows half-maximal saturation at 15–20 μM tyrosine for both young (7–8 days) and older (14–21 days) cultures of neurons (Fig. 1 B,C). It should be pointed out that although the radioactive intracellular tyrosine pool rapidly reaches a steady state and expands in proportion to the external tyrosine concentration (text and Fig. 1 A), free tyrosine represents a small percentage of the total radioactivity taken up and accumulated by the neurons in an 8-h period (about 10%; Fig. 2).

The data on the tyrosine concentration dependence of catecholamine production (Fig. 1 C) agrees well with the work of Levitt et al. (1965). In their study of catecholamine metabolism in the rat heart, rapid perfusion was employed in order to maintain the external tyrosine concentration; 60 μM tyrosine was the saturating value in their study.

Tyrosine Metabolism: Time Dependence

The time course of utilization of tyrosine (text and Fig. 2) shows that under the incubation conditions employed, the intracellular free tyrosine pool of the neurons reaches a steady state of labeling in less than 1 h while the accumulation of radioactive tyrosine (largely in protein) continues linearly for at least 28 h. This indicates that the data from the 8 h incubations normally employed represented ongoing metabolic activity. Rapid and complete equilibration of extracellular and intracellular tyrosine is implied by the following results: (*a*) the intracellular tyrosine pool labels completely within 1 h (text and Fig. 2), (*b*) the radioactive catecholamines synthesized from tyrosine chase very rapidly (Fig. 6 and Table IV), and (*c*) the tyrosine pool expands in response to changes in extracellular tyrosine concentration (Fig. 1 A). The data can only put a bound on the time for the intracellular free tyrosine to reach a steady state of labeling; the half-time must be less than 10 min. The breakdown of unlabeled proteins could contribute unlabeled tyrosine to the intracellular free tyrosine pool, lowering its specific activity with respect to the specific activity of [^{3}H]tyrosine in the medium.

Phenylalanine as Precursor of the Catecholamines

The longstanding assumption that tyrosine is the precursor for catecholamine synthesis has been called into question by recent observations that purified tyrosine hydroxylase (Shiman et al., 1971) as well as brain homogenates (Karobath and Baldessarini, 1972) can convert phenylalanine and tyrosine into catechols. Which precursor is actually

used by sympathetic neurons was investigated in the neuronal cultures. The results of these experiments (Fig. 3) show that the neurons could utilize phenylalanine as a catecholamine precursor, but that at blood levels of tyrosine and phenylalanine, the latter accounted for only 1–2% of the catecholamines produced. $DMPH_4$ reduces the inhibition of tyrosine hydroxylase by tyrosine, and thus could have caused the phenylalanine contribution to the catecholamines synthesized to be abnormally small in the cultures (Shiman et al., 1971). However, deletion of $DMPH_4$ did not change the results. Even under conditions where phenylalanine uptake was 7.5 times as great as tyrosine, the former accounted for less than 10% of the catecholamines synthesized. The possibility that the unlabeled phenylalanine pool was very much larger than the tyrosine pool, thereby isotopically diluting the [^{3}H]phenylalanine much more than the [^{14}C]tyrosine, seems unlikely in view of Eagle's and others' studies (Eagle et al., 1957; Piez and Eagle, 1958) showing that the free tyrosine and phenylalanine pools are very similar in size in other cell types in culture.

The apparent differences between tyrosine hydroxylase prepared from adrenal medulla and from brain (Nagatsu et al., 1971) make it desirable that intact cells from other sources be examined before general conclusions can be drawn concerning the role of phenylalanine in catecholamine metabolism.

Nutritional Factors: Pteridine Cofactors

Studies on purified tyrosine hydroxylase led to the hypothesis that the concentration of its cofactor, biopterin, or the level of the enzyme(s) which reduce(s) the biopterin, play a critical role in controlling the levels of catecholamines in the sympathetic nervous system (Mussachio et al., 1971). This suggestion, coupled with the dearth of knowledge concerning the synthesis, storage, and breakdown of biopterin in higher organisms, led to a number of experiments designed to investigate the effect of added pteridines on catecholamine synthesis by the cultured neurons. These efforts resulted in a small but consistent stimulation by added biopterin on catecholamine synthesis (Table I). The synthetic analog of biopterin, $DMPH_4$, had a similar effect in these experiments. These results suggest that the cofactor was entering the cells and exerting a small effect; they do not provide support for the hypothesis that biopterin levels limit the rate of catecholamine synthesis, nor for the possibility that higher biopterin levels may relieve feedback inhibition of NE on tyrosine hydroxylase in whole cells. It should be noted, however, that the serum in the culture medium or the neurons themselves may provide an excess of biopterin. A recent report (Fukushima and Shiota, 1973) indicates that some mammalian cells can make biopterin from guanine. The recent work of Craine et al. (1972) indicates that high evels of bi opterin reductase are present in various tissues, so that biopterin itself need be present only in small quantities to be effective.

Thoa et al. (1971) reported a stimulation of DA synthesis by $DMPH_4$, but their experimental manipulations abolished NE synthesis in the preparation; the magnitude of the stimulation of DA production by $DMPH_4$ in their experiments was very similar to our data in Table I. Finally, brief reports of a stimulation by biopterin on catecholamine accumulation in sympathetic explants have appeared (Benitez et al., 1970), but these effects were not quantitated, so that comparison with the present results is not possible.

Nutritional Factors: Ascorbic Acid

Ascorbic acid is required for the full hydroxylation of proline and lysine residues during collagen synthesis and secretion in culture (Levenson, 1969; Peterkofsky, 1972 *a*). In addition, a lack of ascorbate can reduce the overall rate of collagen production (Peterkofsky, 1972 *b*). Ascorbic acid also stimulates steroid synthesis in adrenal cells in culture (Sato and Buonassisi, 1964).

It was apparent in these and other studies (Peterkofsky, 1972 *a*; Mohlberg and Johnson, 1963) that ascorbic acid added to culture media is very unstable. These experiments confirmed this instability in L-15. Reduced ascorbate decayed to undetectable levels in about 2 h. This information was then applied in studies of catecholamine synthesis in the cultures. Since the enzyme dopamine-β-hydroxylase utilizes ascorbate to convert DA to NE (Friedman and Kaufman, 1965), it was natural to ask whether the neurons in culture would be affected by frequent additions of fresh ascorbic acid. Neuroblastoma cells, for example, contain substantial amounts of dopamine-β-hydroxylase activity (Anagnoste et al., 1972) yet fail to accumulate NE from their intracellular DA (Schubert et al., 1969). The striking change in the NE/DA ratio brought about by the addition of fresh ascor-

bate to the SCG cultures (Table III) emphasizes this nutritional requirement of sympathetic neurons. These results raise the possibility of using the ascorbate effect to bring the neurotransmitter content of the neurons under experimental control during further electrophysiological and biochemical investigations.

Since ascorbic acid is so unstable, it is not obvious how the neurons can synthesize any NE at all after several weeks in culture (Table III B) without addition of fresh ascorbate. This may be similar to the question why hydroxyproline is found in collagen produced by cells cultured without ascorbic acid (Peterkofsky, 1972 *a*; Woessner and Gould, 1957). Possibly the cells are capable of utilizing another molecule as a reduction cofactor, or of storing small amounts of ascorbate for a long time. In the case of dopamine-β-hydroxylase, the study of Levin and Kaufman (1961) indicates that dopamine itself may provide reducing power under certain circumstances.

Catecholamine Metabolism: Time Dependence

The time course of labeling the catecholamine pools was more complicated than seen for tyrosine. There was a difference in labeling patterns between young (7–8-day) and older (13–14-day) cultures as well as a difference between DA and NE. The DA pool labeled very quickly and the level remained constant throughout the 28-h incubation period. The labeling of the DA pool showed the same form for both young and older cultures (Fig. 4 and text). However, not only was the rate of labeling of the DA pool faster in the older cultures, but the extent or saturation level was greater as well. The half-times of disappearance of radioactive DA (Fig. 6 and Table IV) were consistent with the rates of labeling the pool; the rate of disappearance of the labeled DA in the older cultures was about twice as fast as in younger cultures. These half-times of filling and chasing appear to give the steady state rates of synthesis and breakdown of the DA pool. The failure of DA to accumulate progressively is consistent with a role as a metabolic intermediate in the biosynthetic pathway for NE. However, the labeling patterns reported here may also be explained by the existence of DA-containing cells which are either a small minority in the cultures, or are exhibiting abnormally rapid DA turnover (Bjorklund et al., 1970). Small intensely fluorescent cells have a very stable catecholamine pool in vivo (Norberg et al., 1966) and may contain DA (Bjorklund et al., 1970) or NE (Eranko and Eranko, 1971). Small intensely fluorescent cells have not been detected morphologically in these cultures.

The time course of NE labeling depended on the extent of pretreatment of the cultures with ascorbic acid (Fig. 5), which may mean that labeling depends on the size of the endogenous NE pool at the start of an incubation with radioactive tyrosine. Without ascorbate pretreatment (but incubating in the presence of it), label appeared as NE quickly and the curve continued to rise rapidly for at least 28 h (Fig. 4; Fig. 5 B, open circles). On the other hand, when cultures were fed fresh ascorbate extensively before the incubation, the NE labeling began as before but the rate decreased gradually (Fig. 5 B, closed circles). This difference in labeling pattern is explained by the hypothesis that the ascorbate pretreatment resulted in a large NE pool (unlabeled) before the incubation with radioactive tyrosine. One consequence of a large pool could be that the number of vesicles available for storage of newly made NE would be decreased; thus the initial rate of synthesis of [^{3}H]NE could be the same in the two cases (representing soluble or not yet vesicularized NE), but the ongoing rate (requiring storage of NE) would be decreased. Tyrosine hydroxylase may also be feedback inhibited by NE (Nagatsu et al., 1972). Investigation of this hypothesis awaits determination of the total NE content under these various conditions.

While the synthesis of NE was markedly altered by ascorbic acid pretreatment, the rate at which labeled NE was chased was not affected by prior ascorbic acid additions (Table IV). Rather, the rate of chase of labeled NE was markedly dependent on the age of the culture. As was the case with DA, NE labeled faster and chased faster in older cultures (Table IV and Figs. 6 and 7). Other experiments (unpublished) indicate that 3- and 4-wk old cultures have the same turnover rates as 2-wk cultures. The lack of effect of ascorbate pretreatment on the half-time of NE chases suggests the possibility that the NE breakdown rate is independent of the size of the stores. Direct tests of these possibilities depend on analysis of the total NE content of the cultures. It is hoped that future work, for example involving subcellular fractionation to study the various pools of catecholamine (for reviews, see Hall, 1972; Molinoff and Axelrod, 1971), will clarify the relationship between the rates of synthesis, accumulation, and disappearance reported here.

The rate of disappearance of NE ($t_{1/2}$ = 1.1 h for 2-wk old cultures) was rapid in comparison to most such rates found in vivo, though the results in vivo are quite dependent on the experimental approach, the source of neurons, and the amount of electrical activity the neurons experience (Costa et al., 1972; Spector et al., 1972; Hedqvist and Stjarne, 1969; Sedvall et al., 1968; and Gutman and Weil-Malherbe, 1966). Different techniques have led to widely variant values for the turnover time of NE (in the vasculature and heart) such as 1–3 h and 10–15 h. Lack of electrical activity in endings can reduce the NE turnover rate from 10- to 70-fold. However, the consensus of in vivo studies at this time appears to give half-times of NE turnover in peripheral sympathetic endings of 10–15 h, and in the central nervous system of 2–3 h. Sympathetic ganglia give an even shorter half-time, 1–1.5 h (Brodie et al., 1966; Bhatnagar and Moore, 1971).

Thus, there is some question as to which of these values is the most appropriate comparison for these cultures. Since the cultures contain both cell bodies and fine processes, perhaps the brain is the closest model, though the neurons are obviously of different origins. The SCG (containing primarily cell bodies) may not be the proper model, since sympathetic endings contain the vast majority of neuronal NE in vivo (Dahlstrom and Haggendal, 1966). However, the level of spontaneous electrical activity in the culture (which is unknown at present) would determine how much the endings contribute to the overall NE turnover in culture. Finally, there is also the possibility that in these low density cultures of dissociated neurons the reuptake of neurotransmitter released by spontaneous activity is very poor due to the large extracellular space; in vivo inhibition of NE reuptake during electrical stimulation can increase the NE chase rate by as much as tenfold (Hedqvist and Stjarne, 1969). It may be that the presence of target or other non-neural cells is required for effective reuptake by the neurons. Finally, immature sympathetic endings in vivo have a half-time for NE of about 1 h (Iversen et al., 1967), and it may be that the cultures will mature further with respect to catecholamine turnover beyond the 3-wk period examined here. Thus much more information is needed before the chase rates presented in this paper can be properly evaluated.

The higher rates of synthesis, disappearance, and levels of accumulation of catecholamines in the older cultures (which contain the same number of neurons as young cultures, Mains and Patterson, 1973 *a*) may be of interest in terms of the development of sympathetic neurons. The following paper (Mains and Patterson, 1973 *b*) considers these and other changes in more detail.

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REFERENCES

ANAGNOSTE, B., L. S. FREEDMAN, M. GOLDSTEIN, J. BROOME, and K. FUXE. 1972. Dopamine-β-hydroxylase activity in mouse neuroblastoma tumors and in cell cultures. *Proc. Natl. Acad. Sci. U.S.A.* **69**:1883.

BAKER, H., and O. FRANK. 1968. Clinical Vitaminology. Interscience Publishers Inc., New York.

BALL, E. G. 1937. Studies on oxidation-reduction. XXIII; Ascorbic Acid. *J. Biol. Chem.* **118**:219.

BENITEZ, H. H., L. COTÉ, and M. R. MURRAY. 1970. Regulation of norepinephrine synthesis in organotypic cultures of sympathetic ganglia. *J. Cell Biol.* **47**(2, Pt. 2):18 *a*. (Abstr.).

BHATNAGAR, R. K., and K. E. MOORE. 1971. Effects of electrical stimulation, α-methyltyrosine and desmethylimipramine on the norepinephrine contents of neuronal cell bodies and terminals. *J. Pharmacol. Exp. Ther.* **178**:450.

BJORKLUND, A., L. CEGRELL, B. FALCK, M. RITZEN, and E. ROSENGREN. 1970. Dopamine-containing cells in sympathetic ganglia. *Acta Physiol. Scand.* **78**:334.

BRODIE, B. B., E. COSTA, A. DLABAC, N. H. NEFF, and H. H. SMOOKLER. 1966. Application of steady state kinetics to the estimation of synthesis rate and turnover time of tissue catecholamines. *J. Pharmacol. Exp. Ther.* **154**:493.

COSTA, E., A. R. GREEN, S. H. KOSLOW, H. F. LEFEVRE, A. V. REVUELTA, and C. WANG. 1972. Dopamine and norepinephrine in noradrenergic axons: A study in vivo of their presursor product relationship by mass fragmentography and radiochemistry. *Pharmacol. Rev.* **24**:167.

CRAINE, J. E., E. S. HALL, and S. KAUFMAN. 1972. The isolation and characterization of dihydropteridine reductase from sheep liver. *J. Biol. Chem.* **247**: 6082.

DAHLSTROM, A., and J. HAGGENDAL. 1966. Some quantitative studies on the noradrenaline content in cell bodies and terminals of a sympathetic

adrenergic neuron system. *Acta Physiol. Scand.* **67:** 271.

EAGLE, H. 1955. The specific amino acid requirements of a mammalian cell line (Strain L) in tissue culture. *J. Biol. Chem.* **214:**839.

EAGLE, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science (Wash. D. C.).* **130:**432.

EAGLE, H., K. A. PIEZ, and R. FLEISCHMAN. 1957. The utilization of phenylalanine and tyrosine for protein synthesis by human cells in tissue culture. *J. Biol. Chem.* **228:**847.

ERANKO, O., and L. ERANKO. 1971. Small, intensely fluorescent, granule-containing cells in the sympathetic ganglion of the rat. *Prog. Brain Res.* **34:**39.

FRIEDMAN, S., and S. KAUFMAN. 1965. 3,4-Dihydroxyphenylethylamine β-hydroxylase: physical properties, copper content, and role of copper in the catalytic activity. *J. Biol. Chem.* **240:**4763.

FUKUSHIMA, T., and T. SHIOTA. 1973. Biosynthesis of biopterin, the pteridine cofactor for the hydroxylation of aromatic amino acids, by Chinese hamster ovary (K1) cell cultures. *Fed. Proc.* **32:** Abstr. 2110.

GUTMAN, Y., and H. WEIL-MALHERBE. 1966. Kinetics of catecholamine release by tyramine in rat heart, spleen, and uterus. *Life Sci.* **5:**1293.

HALL, Z. W. 1972. Release of neurotransmitters and their interaction with receptors. *Annu. Rev. Biochem.* **41:**925.

HEDQVIST, P., and L. STJARNE. 1969. The relative role of recapture and of de novo synthesis for the maintenance of neurotransmitter homeostasis in noradrenergic nerves. *Acta Physiol. Scand.* **76:**270.

IVERSEN, L. L., J. DECHAMPLAIN, J. GLOWINSKI, and J. AXELROD. 1967. Uptake, storage and metabolism of norepinephrine in tissues of the developing rat. *J. Pharmacol. Exp. Ther.* **157:**509.

KAROBATH, M., and R. J. BALDESSARINI. 1972. Formation of catechol compounds from phenylalanine and tyrosine with isolated nerve endings. *Nat. New Biol.* **236:**206.

LEVENSON, G. E. 1969. The effect of ascorbic acid on monolayer cultures of three types of chondrocytes. *Exp. Cell Res.* **55:**225.

LEVIN, E. Y., and S. KAUFMAN. 1961. Studies on the enzyme catalyzing the conversion of 3,4-dihydroxyphenylethylamine to norepinephrine. *J. Biol. Chem.* **236:**2043.

LEVITT, M., S. SPECTOR, A. SJOERDSMA, and S. UDENFRIEND. 1965. Elucidation of the rate-limiting step in norepinephrine biosynthesis in the perfused guinea-pig heart. *J. Pharmacol. Exp. Ther.* **148:**1.

LLOYD, T., T. MORI, and S. KAUFMAN. 1971. 6-Methyltetrahydropterin: Isolation and identification as the highly active hydroxylase cofactor from tetrahydrofolate. *Biochemistry.* **10:**2330.

LONG, C., editor 1961. *Biochemists' Handbook.* E. & F. N. Spon, Ltd., London.

MAINS, R. E., and P. H. PATTERSON. 1973 *a*. Primary cultures of dissociated sympathetic neurons. I. Establishment of long-term growth in culture and studies of differentiated properties. *J. Cell Biol.* **59:**329.

MAINS, R. E., and P. H. PATTERSON. 1973 *b*. Primary cultures of dissociated sympathetic neurons. III. Changes in metabolism with age in culture. *J. Cell Biol.* **59:**361.

MEISTER, A. 1965. Biochemistry of the Amino Acids. Academic Press, Inc., New York.

MOHLBERG, J., and M. J. JOHNSON. 1963. Stability of vitamins in a chemically defined medium for 929-L fibroblasts. *J. Natl. Cancer Inst.* **31:**603.

MOLINOFF, P. B., and J. AXELROD. 1971. Biochemistry of catecholamines. *Annu. Rev. Biochem.* **40:**465.

MUSACCHIO, J. M., G. L. D'ANGELO, and C. A. MCQUEEN. 1971. Dihydropteridine reductase: Implication on the regulation of catecholamine biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* **68:**2087.

NAGATSU, T., K. MIZUTANI, I. NAGATSU, S. MATSUURA, and T. SUGIMOTO. 1972. Pteridines as cofactor or inhibitor of tyrosine hydroxylase. *Biochem. Pharmacol.* **21:**1945.

NAGATSU, T., Y. SUDO, and I. NAGATSU. 1971. Tyrosine hydroxylase in bovine caudate nucleus. *J. Neurochem.* **18:**2179.

NORBERG, K. A., M. RITZEN, and U. UNGERSTEDT. 1966. Histochemical studies on a special catecholamine-containing cell type in sympathetic ganglia. *Acta Physiol. Scand.* **67:**260.

PAULING, L. 1970. Vitamin C and the Common Cold. W. H. Freeman and Company Publishers, San Francisco.

PETERKOFSKY, B. 1972 *a*. The effect of ascorbic acid on collagen polypeptide synthesis and proline hydroxylation during the growth of cultured fibroblasts. *Arch. Biochem. Biophys.* **152:**318.

PETERKOFSKY, B. 1972 *b*. Regulation of collagen secretion by ascorbic acid in 3T3 and chick embryo fibroblasts. *Biochem. Biophys. Res. Commun.* **49:**1343.

PIEZ, K. A., and H. EAGLE. 1958. The free amino acid pool of cultured human cells. *J. Biol. Chem.* **231:**533.

ROE, J. H. 1954. Chemical determination of ascorbic, dehydroascorbic, and diketogulonic acids. *Methods Biochem. Anal.* **1:**115.

SATO, G., and V. BUONASSISI. 1964. Hormone synthesis in dispersed cell cultures. *Wistar Inst. Symp. Monogr.* **1:**27.

SCHUBERT, D., S. HUMPHREYS, C. BARONI, and M. COHN. 1969. In vitro differentiation of mouse neuroblastoma. *Proc. Natl. Acad. Sci. U.S.A.* **64:**316.

SEDVALL, G. C., V. K. WEISE, and I. J. KOPIN. 1968. The rate of norepinephrine synthesis measured in vivo during short intervals: influence of adrenergic nerve impulse activity. *J. Pharmacol. Exp. Ther.* **159:**274.

SHIMAN, R., M. AKINO, and S. KAUFMAN. 1971. Solubilization and partial purification of tyrosine hydroxylase from bovine adrenal medulla. *J. Biol. Chem.* **246**:1330.

SPECTOR, S., J. TARVER, and B. BERKOWITZ. 1972. Effects of drugs and physiological factors in the disposition of catecholamines in blood vessels. *Pharmacol. Rev.* **24**:191.

THOA, N. B., D. G. JOHNSON, I. J. KOPIN, and N. WEINER. 1971. Acceleration of catecholamine formation in the guinea pig vas deferens after hypogastric nerve stimulation: roles of tyrosine hydroxylase and new protein synthesis. *J. Pharmacol. Exp. Ther.* **178**:442.

WOESSNER, J. F., and B. S. GOULD. 1957. Collagen biosynthesis: tissue culture experiments to ascertain the role of ascorbic acid in collagen formation. *J. Biophys. Biochem. Cytol.* **3**:685.