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CATECHOLAMINE METABOLISM IN BRAIN SLICES

DETERMINATION OF RELEVANT PRECURSOR POOL AND THE EFFECTS OF ELEVATED K^+

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Abstract—Catecholamine synthesis from [^{3}H]tyrosine was studied in slices of striatum, cerebellum and substantia nigra of mice. If low concentrations of tyrosine (less than 5.5 μM) were added to the incubation medium, the slices released significant amounts of tyrosine into the medium during the incubation. Kinetic analysis of the same experiments indicated that medium tyrosine and not tissue tyrosine was the appropriate precursor for both dopamine (DA) synthesis and protein synthesis in striatal slices. Concentrations of medium tyrosine of 8.25 μM or greater were sufficient to prevent changes of medium tyrosine during incubation and thus maintained a constant specific activity of precursor. Increasing concentrations of medium K^+ increased both the accumulation of [^{3}H]DA and its release from striatal slices. However, accumulation was stimulated at a concentration of K^+ (14 mM) that had no significant stimulatory effect on release, suggesting that the stimulatory effects of K^+ on synthesis and release are mediated by separate processes. Release of $^{14}CO_2$ from [1-^{14}C]tyrosine closely paralleled the accumulation of [^{3}H]DA from [^{3}H]tyrosine. Release of preloaded [^{14}C]DA closely paralleled that of [^{3}H]DA synthesized from [^{3}H]tyrosine, suggesting a common functional pool. The principal DA catabolite produced was dihydroxyphenylacetic acid (DOPAC). The appearance of labeled DOPAC in the media was greatly enhanced by K^+ stimulation.

A variety of drug and hormone treatments has been shown to affect brain catecholamine (CA)† metabolism *in vivo* (e.g. Ref. 1). To study whether such effects are direct or indirect, and to elucidate mechanisms, *in vitro* approaches are needed. The most intact of the *in vitro* preparations is the brain slice. Therefore, as a preliminary to our studies of the effects of various peptide hormones and drugs on CA metabolism *in vitro*, we have characterized the basal metabolism of slices from three brain regions—striatum, cerebellum and substantia nigra. We chose to study the synthesis of catecholamines from [^{3}H]tyrosine, partly because this paralleled a measure we and others have used *in vivo* [2, 3]. Also, this approach enabled us to measure synthesis, release and catabolism in the same system.

Our pilot studies were conducted according to the methods of Versteeg *et al.* [4]. However, we wished to determine an appropriate medium concentration of tyrosine for our studies, to simplify problems due to potential changes in the size of the precursor pool. We also wished to study the effects of different concentrations of K^+ on synthesis and release, because exogenous agents may selectively affect either basal or stimulated rates. Finally, we wished to characterize the nature of the labeled material released from the slices and to identify the principal catabolites.

METHODS

Materials. Male CD-1 mice (Charles River Laboratories, Wilmington, MA, 25–30 g) were used in all experiments; they were housed individually for the 3 days immediately prior to killing. Animals were maintained on a 7:00 a.m. to 7:00 p.m. lighting schedule.

Radioactive materials used were: [2, 6-^{3}H]tyrosine (34 Ci/mmole, Amersham-Searle Corp., Arlington Heights, IL), [1-^{14}C]tyrosine (58 mCi/mmole, New England Nuclear Corp., Boston, MA), and [1-^{14}C]dopamine hydrochloride (55 mCi/mmole, Amersham-Searle).

Procedures. Mice were killed by decapitation. Brains were removed and striatum, substantia nigra, and cerebellum were dissected on a chilled surface. Substantia nigra was obtained by the method of Westerink and Korf [5]. A coronal slab was removed by sectioning at the border of the pons and mesencephalon and at a plane 0.8 mm rostral to it. Substantia nigra, visible near the ventral surface of the slab, was removed along with the ventral tegmental nucleus (A10) from the rest of the mesencaphalon. The weight of this tissue, pooled from both sides, was approximately 2 mg and included

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† Abbreviations used: CA, catecholamine; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; EGTA, ethyleneglycolbis(amino-ethylether)tetra-acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HVA, homovanillic acid; MAO, monoamine oxidase; NE, norepinephrine; PPD, 2,5-diphenylopazole; POPOP, 1,4-bis[2-(4-methyl-5-phenylopazolyl)]benzene; and TH, tyrosine hydroxylase.

ventral portions of the mesencaphalic reticular formation. In a typical fifty-sample experiment, ten, twenty, or forty mice were used to generate slices of cerebellum, striatum, or substantia nigra respectively. Dissected pieces were sliced in two dimensions with a McIlwain tissue chopper set at 0.3 mm. They were then pooled and suspended in medium at 37° in an O_2:CO_2 (95:5, v/v) atmosphere in a small (125 ml) Erlenmeyer flask. The medium used in all experiments was that described by Versteeg *et al.* [4] and contained the following: 118 mM NaCl, 4.4 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM K_2HPO_4, 25 mM HEPES (Ultrol, Calbiochem-Behring Corp., La Jolla, CA), and 12 mM glucose titrated to pH 7.3 with NaOH. Increases in KCl were osmotically balanced with equivalent decreases in NaCl. After 30 min, the medium was aspirated and replaced with fresh, equilibrated medium at 37° for an additional 10 min. This preparation is referred to as equilibrated slices. Incubations were performed in a Lab-Line Orbit Water Bath Shaker (model 3525) which kept the slices in suspension in the medium at all times. A stainless steel manifold secured to the shaking part of the bath enabled delivery of O_2:CO_2 separately to each incubation tube via PE-50 cannula tubing. This arrangement made it possible to manipulate tubes individually without disturbing the others.

Three kinds of experiments were performed.

First, the accumulation of ^{3}H-labeled catecholamines from [^{3}H]tyrosine was measured. Equilibrated slices (200–500 μg protein in 0.5 ml) were added to a series of Corex round-bottomed 15 ml centrifuge tubes along with 0.5 ml of equilibrated medium containing [2, 6-^{3}H]tyrosine. Total incubation volume was 1.0 ml. Pilot experiments indicated that [^{3}H]CA accumulations were independent of this volume, in the range 0.2 to 2.0 ml, and proportional to tissue weight in the range 0.1 to 2 mg protein. Samples were incubated for 40 min. The reactions were terminated by the addition of 2 ml of ice-cold medium and centrifuged at 100 *g* (400 rpm) in a Sorvall RC-3 centrifuge at 4°. Media were decanted and in some instances saved and processed for measurement of released radioactive metabolites. A further 2 ml of ice-cold medium was added to the samples, and the centrifugation was repeated. The supernatant fraction was decanted and discarded.

Second, the liberation of $^{14}CO_2$ from [1-^{14}C]tyrosine was measured. Slices and media were prepared as in the accumulation experiments, except that [1-^{14}C]tyrosine was used in place of [^{3}H]tyrosine. After the slices were added to equilibrated media containing label, a plastic cup (Kontes Glass Co. Vinland, NJ) filled with glass wool, moistened with 0.05 ml Soluene 350 (Packard Instrument Co. Downer's Grove, IL) and suspended from a rubber stopper was inserted into the Corex centrifuge tube. After 20 min of incubation, the reaction was stopped by injecting 0.1 ml of 50% trichloroacetic acid through the rubber stopper. After standing overnight at 4°, each plastic cup was removed and placed in a scintillation vial with 10 ml of scintillator (0.35% PPO–0.01% POPOP in toluene).

Third, the release and metabolism of labeled DA from preloaded slices were studied. Pooled slices were exposed to [^{3}H]tyrosine (25 μCi/ml medium; 750 nM; typically 30 ml total volume) for 10 min. [^{14}C]DA (1 μCi/ml medium; 18 μM) was added, and the slices were incubated for a further 10 min with both labeled substrates. Slices were then washed four times with fresh equilibrated medium. Slices preloaded in this manner with [^{3}H]- and [^{14}C]DA were added in a volume of 0.5 ml to Corex centrifuge tubes containing 0.5 ml of equilibrated medium. Slices were incubated for 10 min, and the reaction was stopped with 1 ml of ice-cold medium. Tissue and media were separated as described above.

Chromatography. Purification procedures were essentially those of Iuvone *et al.* [6]. Rinsed slices were homogenized in 2.0 ml of 0.4 M perchloric acid, containing 0.05% sodium metabisulfite in the Corex centrifuge tubes using a Teflon pestle machined to a diameter of 14.5 mm to fit the tubes tightly. The homogenate was centrifuged at 15,000 *g* in a refrigerated (4°) Sorvall RC-2B centrifuge. This arrangement permitted incubation, centrifugation and homogenization, all to be conducted in the same tube, thus minimizing errors due to losses during transfer.

Supernatant fractions were loaded onto AG50W-X4 (Bio-Rad Laboratories, Richmond, CA) cation-exchange columns (6 × 20 mm, H^+-form). The load volume, together with a 2.0 ml wash with sodium acetate buffer (0.1 M sodium acetate–0.1% disodium EDTA, titrated to pH 7.0 with 1 M NaOH) was collected in test tubes containing 200 mg of alumina [7], moistened with 0.1 ml of 0.1 M disodium EDTA. Samples were titrated with approximately 0.5 ml of 3 M Tris hydroxide (Trizma base, Sigma) to a pH of 7.9 and vortexed for 5 min. The alumina suspension was transferred to a glass-wool stoppered Pasteur pipette. The fraction not bound to the alumina, along with 0.5 ml of a water wash, was collected in a scintillation vial containing 10 ml of a Triton scintillator [1.07% PPO–0.012% POPOP in toluene, mixed 1:1.4 with Triton X-100 (Scintillar, Mallinckrodt Laboratory Chemicals, Paris, KY)]. This fraction contained ^{3}H-water and methylated, deaminated metabolites of DA which do not bind to alumina. The alumina was washed with another 5.5 ml of water, and then catechols were eluted with 2.0 ml of 0.5 M HCl into scintillation vials and counted in 10 ml of Triton scintillator. More than 90% of the label in this fraction was determined to be DOPAC by paper chromatography in *n*-butanol–acetic acid–H_2O (120:30:50, by vol.)

The cation-exchange columns were then washed with an additional 6 ml of sodium acetate buffer. Tyrosine was eluted with a second 6 ml of sodium acetate buffer. The eluate was adjusted to pH 1.5 with 4 M HCl, and loaded onto standard AG-50W-X4 cation-exchange columns (6 × 20 mm) charged with Na^+. Following washes with 5 ml of water and 8 ml of 0.5 M HCl, tyrosine was eluted with 9 ml of 0.1 M Na_3PO_4. Of this volume, 0.9-ml aliquots were added to 0.1 ml of 4 M HCl and 10 ml of Triton scintillator, while 1.8-ml aliquots were assayed for tyrosine using nitrosonaphthol [8].

After the tyrosine fraction was eluted, the original (H^+-form) columns were washed with 2 ml of sodium acetate buffer and 2 ml of 1 M HCl. NE was then eluted with 4.75 ml of 1 M HCl into test tubes con-

taining 200 mg alumina. Following a further wash with 1.25 ml of 1 M HCl, DA was eluted from the cation-exchange columns with 2.5 ml of 4 M HCl into test tubes containing 200 mg alumina. NE and DA fractions were titrated to pH 7.9 with approximately 2.6 ml of 3 M Tris and approximately 0.5 ml of 10 M NaOH respectively. Preparation, transfer, and filtration of alumina, as well as the elution of NE and DA from alumina, were as described above for the purification of DOPAC.

In experiments in which release from the slices during incubation was analyzed, the media recovered from the first centrifugation were titrated to pH 1.5 with 0.063 ml of 4 M HCl and loaded onto the AG50W-X4 (H^+-form) columns. The elution profile and secondary purifications were identical to those used for the homogenate supernatant fraction, except that the sodium acetate buffer wash preceding the tyrosine elution was shortened to 4 ml instead of 6 ml.

In the release and metabolism experiments, more than 99% of all radioactivity in an incubated tube could be accounted for when the above fractions were summed. Recoveries for each compound were 99% for tyrosine, 85% for Ne, and 75% for DA. Less than 0.01% of [^{3}H]tyrosine and 1% of NE and DA appeared in any of the other fractions. Results are not corrected for recoveries.

The pellets formed by the centrifugation of the homogenate were dissolved in 2.5 ml of 0.3 M NaOH. Protein determinations were performed on duplicate 0.25-ml aliquots [9].

Quantification and analysis. Radioactivity was assayed using a Packard 2425 liquid scintillation spectrophotometer. Correction factors for quenching and for emission spectra overlap in double-label experiments were determined with automatic external standardization.

The dpm for each fraction (except media tyrosine) were divided by the amount of protein in that tissue sample to derive specific activities. Tyrosine specific activity was expressed as dpm/μg tyrosine. Blank values, determined in samples to which radioactivity was added but which were chilled to 0° and not incubated, were subtracted from experimental values. Data for accumulation studies were expressed as moles of CA accumulated per mg protein per min (based on the tyrosine specific activity). Data from metabolism studies were expressed as percentage of total radioactivity recovered in fractions containing labeled DA or its metabolites.

Student's *t*-tests were performed on a Hewlett–

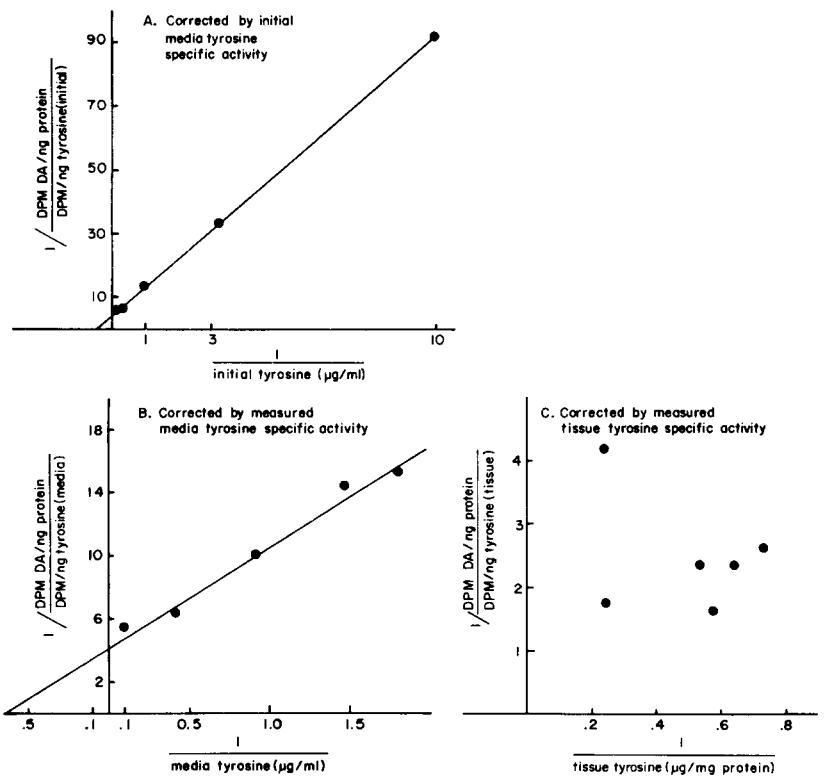

Fig. 1. Kinetics of [^{3}H]DA accumulation from [^{3}H]tyrosine: effects of correcting with initial media, measured media, and measured tissue tyrosine specific activities. Striatal slices were incubated for 30 min with 4.17 μCi/ml [^{3}H]tyrosine at 0.03, 0.1, 0.3, 1, 3, and 10 μg/ml tyrosine (0.17, 0.55, 1.7, 5.5, 17 and 55 μM respectively). DA specific activities (dpm/mg protein) were divided by tyrosine specific activities (dpm/ng tyrosine) and are presented as double-reciprocal plots against tyrosine concentrations (μg/ml medium). Each point is the mean of three determinations. (A) [^{3}H]DA accumulation normalized by initial tyrosine specific activity as a function of initial medium tyrosine concentration. (B) [^{3}H]DA accumulation normalized by measured medium tyrosine specific activity as a function of measured medium tyrosine. (C) [^{3}H]DA accumulation normalized by measured tissue tyrosine specific activity as a function of measured tissue tyrosine concentration.

Packard 9810A calculator. An Amdahl 370, version 6 computer was used for quench and double-label corrections.

RESULTS

Determination of relevant precursor pool. Striatal slices were incubated in media containing various concentrations of tyrosine as described in the legend in Fig. 1. Measurements of [^{3}H]DA accumulation (dpm/μg protein) were divided by the initial (calculated) medium tyrosine specific activities. When plotted as a function of initial medium tyrosine concentration on a double-reciprocal plot, normal Michaelis–Menten kinetics were observed (Fig. 1A). However, because tyrosine was being exchanged between the medium and the slices, the specific activities of both medium and tissue tyrosine during the incubation would be somewhat different from the initial specific activities. Thus, we measured the specific activities of tyrosine in the tissue and medium after incubation at each initial concentration of tyrosine and repeated the kinetic analyses. The measured concentrations of media tyrosine were markedly higher than those originally added to the media when the initial concentration was less than 5.5 μM. This indicated that tyrosine was being released from the tissue, diluting the medium tyrosine specific activity (Table 1). Conversely, initial media tyrosine concentrations of more than 5.5 μM elevated tissue tyrosine concentrations.

The DA specific activity was normalized by either the measured media or the measured tissue tyrosine specific activity and subsequently plotted on double-reciprocal plots as functions of measured media or measured tissue tyrosine concentrations respectively. Despite the narrowing of the range of tyrosine concentrations in the media, the data corrected with the media tyrosine specific activities displayed Michaelis–Menten kinetics (Fig. 1B) with a V_{max} and K_m similar to those determined using the initial tyrosine specific activity. Data normalized with tissue tyrosine specific activity did not display normal kinetics (Fig. 1C). Although not shown, the labeling of protein with [^{3}H]tyrosine also displayed Michaelis–Menten kinetics when media, but not tissue, tyrosine specific activities were used to normalize the data.

Table 1. Relationship of measured medium and measured tissue tyrosine concentration to initial tyrosine concentrations

Added to medium (initial) [μg/ml (μM)]	Tyrosine concentration Measured at end of incubation Medium (μg/ml)	Tissue (μg/mg protein)
0.03 (0.17)	0.58 ± 0.06	1.73 ± 0.40
0.10 (0.55)	0.56 ± 0.12	1.55 ± 0.37
0.30 (1.7)	0.68 ± 0.08	1.36 ± 0.13
1.0 (5.5)	1.09 ± 0.07	1.85 ± 0.49
3.00 (17)	2.40 ± 0.12	4.18 ± 1.19
10.00 (55)	8.98 ± 0.11	4.18 ± 0.58

Since media tyrosine concentrations above 5.5 μM were not appreciably diluted by tyrosine released from the slices, subsequent experiments used 8.3 μM (1.5 μg/ml, the concentration used by Versteeg *et al.* [4]) or 83 μM tyrosine in the medium. At these concentrations, the specific activity of medium tyrosine, the pool which best reflects the relevant precursor, was assumed to be unaltered during incubation.

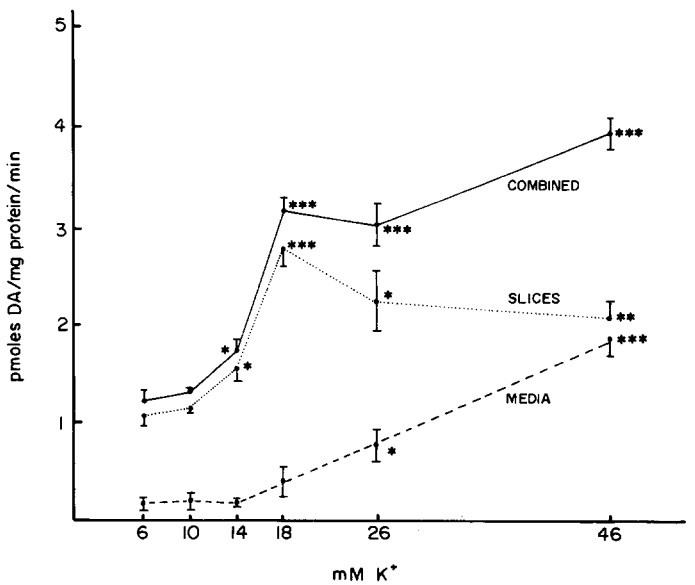

Fig. 2. Effects of elevated K^+ on the accumulation of [^{3}H]DA in striatal slices and media. Striatal slices were incubated with 83 μM tyrosine and [^{3}H]tyrosine (4.17 μCi/ml). Each point is the mean (± S.E.) of four determinations. Combined accumulation is the sum of slice and medium accumulations. Key: (*) significant increase from 6 mM K^+, $P < 0.05$; (**) $P < 0.01$; and (***) $P < 0.001$ (Student's *t*-test).

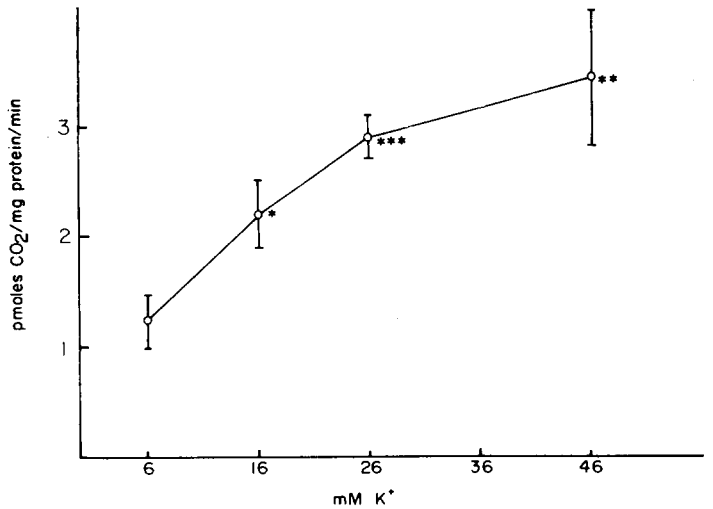

Fig. 3. Facilitation by K^+ of $^{14}CO_2$ liberation from slices incubated with [1-^{14}C]tyrosine. Incubation was for 20 min at 83 nCi/ml. Each point is the mean (± S.E.) of four determinations. Key: (*) significant difference from saline, $P < 0.05$; (**) $P < 0.01$; and (***) $P < 0.001$ (*t*-test).

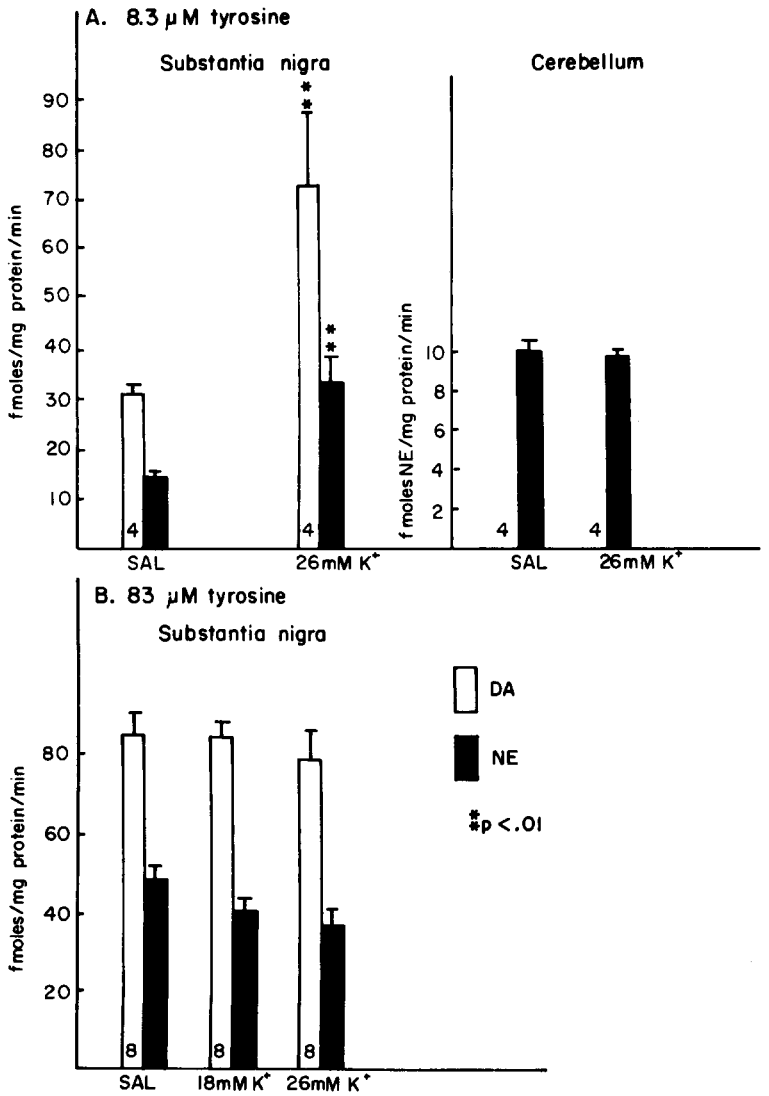

Fig. 4. Effects of elevated K^+ on the accumulation of [^{3}H]DA and [^{3}H]NE in substantia nigral and cerebellar slices. (A) Substantia nigral and cerebellar slices incubated with 4.17 μCi/ml and 16.7 μCi/ml [^{3}H]tyrosine, respectively, at 8.3 μM tyrosine. (B) Substantia nigral slices incubated with 16.7 μCi/ml [^{3}H]tyrosine at 83 μM tyrosine. Numbers at the base of each bar indicate the sample size for each group. SAL indicates standard incubation conditions (6 mM K^+). Key: (*) significant difference from saline ($P < 0.01$).

K^+-induced activation of the accumulation of ^3H-labeled catecholamines. Various concentrations of KCl were added to striatal slices incubated in 83 μM tyrosine containing [^{3}H]tyrosine. [^{3}H]DA was recovered from both slices and media. As shown in Fig. 2, elevated K^+ increased the formation and release of [^{3}H]DA. Combined slice and media [^{3}H]DA accumulations reached a maximum at 18 mM K^+. Interestingly, accumulation of [^{3}H]DA in the slices was increased by 14 mM K^+ which had no significant effect on release. The accumulation of [^{3}H]DA from [^{3}H]tyrosine in striatal slices was elevated 225, 260 and 322% by 26 mM K^+ at 8.3, 83, and 274 μM tyrosine respectively. Elevated K^+ had no significant effect on [^{3}H]tyrosine uptake into slices (data not shown).

The asymptote in [^{3}H]DA accumulation at concentrations of K^+ higher than 18 mM may reflect either a limit on synthesis or concurrent, linear increases in [^{3}H]DA synthesis and catabolism. To test whether the asymptote reflected a limit of synthesis, slices were incubated with [1-^{14}C]tyrosine. The release of $^{14}CO_2$, an index of decarboxylation of tyrosine or tyrosine derivatives, also displayed an apparent asymptotic response at K^+ concentrations equal to or greater than 26 mM. The four points in Fig. 3 are almost identical to the comparable values for combined [^{3}H]DA accumulation in Fig. 2.

A K^+-induced increase in accumulation was not consistently observed in other regions. [^{3}H]NE accumulation from [^{3}H]tyrosine was not increased in cerebellar slices incubated at 8.3 μM tyrosine. In substantia nigral slices, both [^{3}H]NE and [^{3}H]DA accumulations were increased by 26 mM K^+ at a medium tyrosine concentration of 8.3 μM, but not 83 μM (Fig. 4).

K^+-induced activation of the disappearance of preloaded, labeled DA. The effect of 26 mM K^+ on the disappearance of labeled DA from striatal slices was measured. Equilibrated slices were incubated with both [^{3}H]tyrosine and [^{14}C]DA. Following a thorough washing to remove excess radioactivity, 0.5 ml of labeled slices was added to 0.5 ml of unlabeled medium containing combinations of high or low K^+ (6 or 26 mM) and normal Ca^{2+} (2.6 mM) or depleted Ca^{2+} (0.1 mM EGTA with no exogenous Ca^{2+}). Radioactive (^{3}H and ^{14}C) DA in the media, DA remaining in the slices, total (slices and medium combined) DOPAC, and total residual [tritiated water, homovanillic acid (HVA)] radioactivity were measured (Fig. 5).

Slice DA radioactivity inversely reflected the combined changes in media DA and total DOPAC. Under basal conditions (i.e. 6 mM K^+ and 2.6 mM Ca^{2+}), more than 85% of the label in the preparation was recovered as labeled DA remaining in the slices. With the addition of 26 mM K^+, a 25% decrease in slice DA radioactivity was observed. Most of the displaced label was in media DA, but radioactive DOPAC levels increased approximately 2-fold (statistically significant only for ^{3}H).

Under Ca^{2+}-depleted conditions, K^+-stimulated release of labeled DA was reduced drastically, but not eliminated ($P < 0.01$). However, K^+-stimulated accumulation of labeled DOPAC was not reduced by Ca^{2+} depletion.

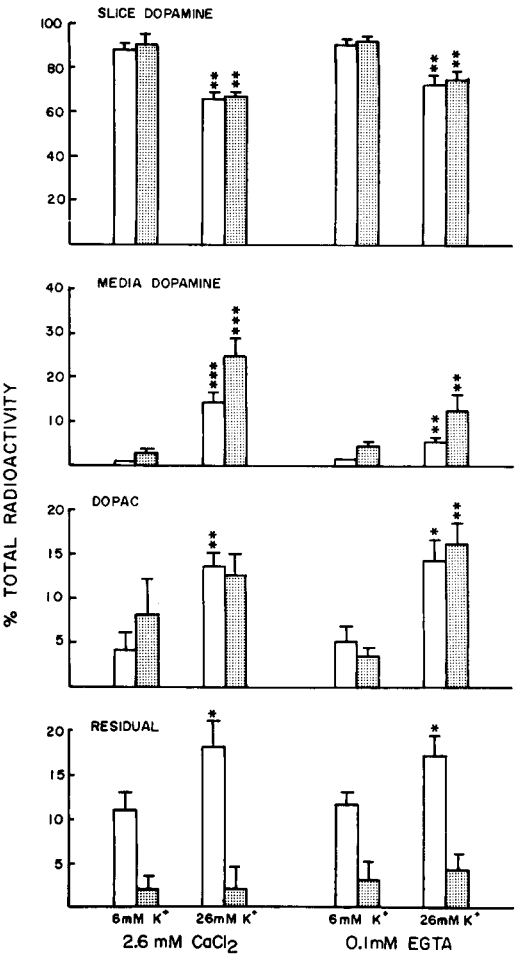

Fig. 5. Effects of 26 mM K^+ and Ca^{2+} depletion on the release and catabolism of labeled DA, preloaded in striatal slices. Ca^{2+} depletion was accomplished with 100 μM EGTA in the absence of exogenous Ca^{2+}. Pooled striatal slices were exposed to [^{3}H]tyrosine (250 μCi/10 ml) for 10 min. Then 10 μCi/10 ml [^{14}C]DA was added, and the slices were incubated for an additional 10 min. Slices were then washed four times. Aliquots of 0.5 ml of slices were added to 0.5 ml of media of appropriate ionic composition and incubated for 10 min. Open and shaded bars represent the distribution of ^{3}H and ^{14}C respectively. DOPAC and residual (containing ^3H_2O and labeled HVA) fractions were pooled from the slices and the media. The sum of slice DA, media DA, DOPAC, and residual equals 100% for each label. Each bar represents the mean and standard error of eight determinations. Key: (*) significant difference between 6 and 26 mM K^+ conditions for the same label under either 2.6 mM or depleted Ca^{2+} conditions, $P < 0.05$; (**) $P < 0.01$; and (***) $P < 0.001$ (Student's *t*-test).

For the most part, the distributions of ^{3}H and ^{14}C were very similar, but much higher percentages of ^{3}H were observed in the residual fraction than was the case for ^{14}C. Small relative decreases of ^{3}H in other fractions, particularly [^{3}H]DA found in the media, balanced the excess ^{3}H in the residual fraction. Since drying this fraction before adding scintillation fluid could eliminate the difference, the excess ^{3}H was assumed to be associated with water.

Residual ^{14}C-radioactivity was not affected significantly by any ionic manipulation. Since HVA is found in this fraction, [^{14}C]HVA does not appear to be a significant metabolite of [^{14}C]DA *in vitro*, especially under K^+-stimulated conditions. However, residual ^3H-radioactivity was increased significantly by 26 mM K^+. This effect may reflect ^3H-water derived from [^3H]DA released by K^+. These studies suggest that DA newly synthesized from tyrosine and that accumulated by reuptake enter a common functional pool.

DISCUSSION

Precursor pool determination. The accumulation of [^3H]DA from [^3H]tyrosine in striatal slices displayed Michaelis–Menten kinetics when the DA specific activities were corrected with either initial or measured *media* tyrosine specific activities. Such was not the case when measured *tissue* tyrosine specific activities were used as correction factors. Kapatos and Zigmond [10] reported similar findings using striatal synaptosomes. These results indicate compartmentation of tissue tyrosine.

If the compartment into which tyrosine is taken up for CA synthesis is small and metabolically active, its tyrosine specific activity would be much higher than the measured (average) tissue specific activity. This compartment might be within the synaptic terminals. It is possible that tyrosine hydroxylase (TH) may be linked to a tyrosine uptake mechanism at the plasma membrane. However, this would only account for DA synthesis. We also found that protein labeling with [^3H]tyrosine in the same slices displayed Michaelis–Menten kinetics only when corrected with media tyrosine specific activities. Once again these data are best explained if one assumes that the volume for protein synthesis, neuronal perikarya, is a relatively small portion of brain tissue. Since this volume is metabolically active, [^3H]tyrosine would quickly equilibrate between the neuronal perikarya and the extracellular space. Other, less metabolically active regions, such as glia and neuropil, may not take up as much [^3H]tyrosine and hence would possess lower specific activities of precursors. Measurements of whole tissue [^3H]tyrosine specific activities would combine such pools making such measurements inaccurate estimates of the specific activity of [^3H]tyrosine in pools available for protein synthesis.

Several radioactive tracer techniques of determining transmitter turnover rates *in vivo* have been proposed which depend upon measured precursor specific activities as a normalizing factor (e.g. Ref. 2). The dissociation of tissue tyrosine specific activity from DA synthesis *in vitro* suggests that measurable tyrosine specific activities (and perhaps other transmitter precursors) *in vivo* may not reflect the relevant precursor pools for synthesis.

Dopamine compartmentation. A variety of evidence has suggested that DA is stored in two metabolically distinct compartments [11]. The initial observation was that inhibition of TH with α-methyl-*p*-tyrosine resulted in a biphasic decline of DA content from striata [12]. The most compelling evidence is that the release of [^3H]DA newly synthesized from [^3H]tyrosine is enhanced under conditions that are relatively ineffective in eliciting the release of [^3H]DA stored for longer periods [13]. Finally, the simultaneous measurement of release and synthesis of [^3H]DA from [3,5-^3H]tyrosine indicates that changes in release and synthesis are parallel, arguing that newly synthesized DA is the pool preferentially released.

However, an important variable in these experiments is the duration of the labeling period. Most experiments that have identified compartments of labeled catecholamines have compared short with long labeling periods. In the present study, the administration of [^{14}C]DA to brain slices occurred during [^3H]tyrosine exposure. The identical rates of [^3H]- and [^{14}C]DA release and catabolism observed (except for $^3H_{20}$ formation from released [^3H]DA) indicate that, although separate compartments may exist, they cannot be distinguished on the basis of their derivation from synthesis or re-uptake. Different labeling periods appear to be the critical factor for identifying different compartments.

Effects of elevated K^+ upon synthesis, release, and metabolism of DA. Depolarizing agents such as K^+ or veratridine induce release and activate synthesis of DA *in vitro*. Both the induction of release and the activation of synthesis are calcium dependent [14, 15]. The increase is correlated with an activation of TH to a form characterized by an increased affinity for reduced pteridine cofactor and a decreased sensitivity to end-product inhibition by DA [16].

It has been suggested that the K^+-induced activation of synthesis is a consequence of increased release of DA and consequent reduced end-product inhibition [10, 17–19]. However, we observed that the activation of [^3H]DA accumulation could be dissociated from the induction of release. First, K^+ concentrations higher than 18 mM produced an asymptotic effect on combined slice and medium [^3H]DA accumulation, while release increased linearly with increasing K^+ concentrations up to at least 46 mM. The increase of $^{14}CO_2$ evolution from [1-^{14}C]tyrosine matched the effects on combined slice and medium [^3H]DA accumulation at 16, 26, and 46 mM K^+. This similarity suggests that the asymptote reflects a true limit of synthesis rate and not combined parallel increases in synthesis and catabolism. The plateau of synthesis activation may reflect an inhibitory feedback upon DA synthesis mediated by released DA which stimulates a presynaptic receptor that inhibits synthesis, or it may reflect maximal activation of available enzymes.

Second, combined slice and medium [^3H]DA was increased significantly at 14 mM K^+ ($P < 0.05$), a concentration at which no effect was detected upon release (i.e. [^3H]DA in medium). However, the possibility that release occurred at 14 mM K^+, but that efficient reuptake and degradation by MAO kept the amount of [^3H]DA in the media minimal, cannot be dismissed. In support of this, K^+-stimulated formation of [^3H]DOPAC was found to be constant, regardless of the quantity of release (normal Ca^{2+} as opposed to Ca^{2+}-depleted conditions, Fig. 5). Thus, reuptake of [^3H]DA into the slices might have been saturated by released DA at K^+ concentrations higher than 14 mM, resulting in a net accumulation of [^3H]DA in the media. At K^+ concentrations below

18 mM, if reuptake had been completely effective in recovering released DA, no [^{3}H]DA would have accumulated in the media. However, K^+-induced release and synthesis were reported previously to be dissociable by Bustos *et al.* [16]; K^+-induced TH activation in striatal slices was suppressed by ethanol, while release was unaffected. Since TH activation by depolarization is calcium dependent [20], synthesis activation may occur subsequent to Ca^{2+} influx into the terminal in a process parallel to, but not necessarily dependent upon, Ca^{2+}-mediated release.

It is curious that K^+ did not increase the accumulation of [^{3}H]DA and [^{3}H]NE in substantia nigral slices at 83 μM tyrosine or of [^{3}H]NE in cerebellar slices. Assuming that increased release of labeled catecholamines occurred during incubation, an increase in synthesis may actually have occurred, but all the [^{3}H]DA was released into the media. However, amphetamine, which appears to activate DA synthesis in a manner similar to K^+, also interacts with tyrosine, being effective only at media concentrations of tyrosine above 10 μM [21]. Also, the effectiveness of apomorphine in inhibiting synthesis is reduced in the substantia nigra at 83 μM tyrosine [22]. It is not at all clear why the effect of K^+ in striatum and the effect of amphetamine in substantia nigra [21] would be potentiated by increasing tyrosine concentrations, while the effect of K^+ would be suppressed at higher tyrosine concentrations in substantia nigral slices.

A significant percentage (20%) of the labeled [^{3}H]DA released by K^+ stimulation was found to be converted almost exclusively to the deaminated, nonmethylated metabolite, DOPAC, as was observed for NE terminals [23] and for striatal slices [24]. Cubeddu *et al.* [24] found that reuptake blockers reduced the formation of DOPAC, suggesting that the DOPAC was derived from labeled DA that had been released, and subsequently taken up, by the terminal. Our finding that the elevation of labeled DOPAC formation, caused by 26 mM K^+, occurred independently of the presence of Ca^{2+} is somewhat at odds with this interpretation. However, if uptake mechanisms were saturated by the DA released by 26 mM K^+ under Ca^{2+}-depleted conditions, then uptake might proceed at nearly the same rate as in media containing Ca^{2+}, thus making the amount of precursor available for MAO fairly constant.

In summary, our most significant findings are as follows: (1) the medium tyrosine specific activity, as opposed to that in the tissue, appeared to reflect that of the relevant precursor pool for both DA and protein syntheses in striatal slices, (2) DOPAC was the principal metabolite of DA found in striatal slices, confirming earlier reports [23, 24], and was apparently the exclusive metabolite of DA released by K^+ stimulation, and (3) the accumulation and release of [^{3}H]DA synthesized from [^{3}H]tyrosine appeared to be dissociable in that increased total accumulation of [^{3}H]DA from tyrosine could be induced by 14 mM K^+, which did not significantly elevate the amount of [^{3}H]DA recovered from the media.

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REFERENCES

1. D. H. G. Versteeg, *Pharmac. Ther.* **11**, 535 (1980).
2. N. H. Neff, P. F. Spano, A. Groppetti, C. T. Wang and E. Costa, *J. Pharmac. exp. Ther.* **176**, 701 (1971).
3. P. M. Iuvone, J. Morasco, R. L. Delanoy and A. J. Dunn, *Brain Res.* **139**, 131 (1978).
4. D. H. G. Versteeg, J. M. van Ree, A. P. Provoost and W. de Jong, *Life Sci.* **15**, 2127 (1974).
5. B. H. C. Westerink and J. Korf, *Eur. J. Pharmac.* **40**, 131 (1976).
6. P. M. Iuvone, J. Morasco and A. J. Dunn, *Brain Res.* **120**, 571 (1977).
7. A. H. Anton and D. F. Sayre, *J. Pharmac. exp. Ther.* **138**, 360 (1962).
8. S. Udenfriend, in *Fluorescence Assay in Biology and Medicine* (Eds. N. O. Kaplan and H. A. Scheraga), p. 130. Academic Press, New York (1962).
9. J. L. Bailey, *Techniques in Protein Chemistry*, 2nd Edn, p. 340. Elsevier, Amsterdam (1967).
10. G. Kapatos and M. J. Zigmond, *J. Neurochem.* **28**, 1109 (1978).
11. J. Glowinski, in *Metabolic Compartmentation and Neurotransmission, Relation to Brain Structure and Function* (Eds. S. Berl, D. D. Clarke and D. Schneider), p. 187. Plenum Press, New York (1975).
12. F. Javoy and J. Glowinski, *J. Neurochem.* **18**, 1305 (1971).
13. M. J. Besson, A. Cheramy, P. Feltz and J. Glowinski, *Brain Res.* **32**, 407 (1971).
14. J. E. Harris and R. H. Roth, *Molec. Pharmac.* **7**, 593 (1971).
15. G. Bustos and R. H. Roth, *Biochem. Pharmac.* **28**, 1923 (1979).
16. G. Bustos, R. H. Roth and V. H. Morgenroth, *Biochem. Pharmac.* **25**, 2493 (1976).
17. A. Alousi and N. Weiner, *Proc. natn. Acad. Sci. U.S.A.* **56**, 1491 (1966).
18. S. Spector, R. Gordon, A. Sjoerdsma and S. Udenfriend, *Molec. Pharmac.* **3**, 549 (1967).
19. R. H. Roth, J. R. Walters and G. K. Aghajanian, in *Frontiers in Catecholamine Research* (Eds. E. Usdin and S. H. Snyder), p. 567. Pergamon Press, Oxford (1973).
20. J. R. Simon and R. H. Roth, *Molec. Pharmac.* **16**, 224 (1979).
21. N. J. Uretsky and R. S. Snodgrass, *J. Pharmac. exp. Ther.* **202**, 565 (1977).
22. R. L. Delanoy and A. J. Dunn, *Biochem. Pharmac.* **31**, 3299 (1982).
23. M. B. Farah, E. Adler-Graschinsky and S. Z. Langer, *Naunyn-Schmiedeberg's Archs Pharmac.* **297**, 119 (1977).
24. L. X. Cubeddu, I. S. Hoffman and G. B. Ferrari, *J. Pharmac. exp. Ther.* **209**, 165 (1979).