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Release of monoamines from the striatum and hypothalamus: effect of γ-hydroxybutyrate

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Summary

1. A simple *in vitro* system was developed to study the effect of γ-hydroxybutyrate on nerve cell depolarization-induced release of labelled dopamine, noradrenaline and 5-hydroxytryptamine from brain slices.

2. The release of ^{3}H-dopamine formed in rat striatal slices incubated with ^{3}H-tyrosine was followed either by transferring the slices through successive media or by using a superfusion system. A one to three minute exposure to K^+ (53 mM) caused up to a thirty fold increase in the release of newly synthesized ^{3}H-dopamine. This K^+-induced release was antagonized when γ-hydroxybutyrate (1×10^{-3}M) was present in the medium.

3. Potassium (53 mM) increased (eighteen to thirty fold) the release of ^{3}H-dopamine from striatal slices initially loaded by preincubation with ^{3}H-dopamine. However, the K^+-induced release of this pool of dopamine was not antagonized by γ-hydroxybutyrate.

4. Potassium (53 mM) also increased the release from striatal slices of ^{3}H-5-hydroxytryptamine newly synthesized from ^{3}H-tryptophan. This K^+-induced release of 5-hydroxytryptamine was also not inhibited by γ-hydroxybutyrate.

5. The release of newly synthesized ^{3}H-noradrenaline from hypothalamic slices was also increased by K^+. This K^+-induced release, however, unlike that of 5-hydroxytryptamine, was antagonized when γ-hydroxybutyrate was present in the superfusion medium.

6. Removal of Ca^{++} had no effect on K^+-induced release of ^{3}H-dopamine when followed by transferring the slices through successive media. This K^+-induced release was abolished, however, when Mg^{++} (12 mM) was present in the medium.

7. The removal of Ca^{++} from the superfusion medium abolished almost completely the K^+-induced release from striatal slices of either newly synthesized ^{3}H-dopamine or preloaded ^{3}H-dopamine. This is presumably due to a more effective washout of tissue Ca^{++} by the superfusion technique.

8. The ability of γ-hydroxybutyrate to antagonize the K^+-induced release of monoamines from brain slices does not appear to be unique to the release of newly synthesized dopamine from the striatum.

Introduction

γ-Hydroxybutyrate (GHB) has unique characteristics among compounds which cause behavioural depression of the nervous system. It occurs as a natural

metabolite of mammalian brain (Roth & Giarman, 1970) and when administered parenterally causes a marked increase in brain dopamine (DA) (Gessa, Vargiu, Crabai, Boero, Caboni & Camba, 1966; Roth & Surh, 1970) with little or no effect on the level of other brain monoamines and γ-aminobutyric acid (Giarman & Schmidt, 1963; Gessa *et al.*, 1966; Roth & Surh, 1970). The increase in brain DA induced by GHB is localized to the striatum and other brain areas rich in DA containing nerve terminals and appears to have the same regional distribution as normal endogenous DA (Gessa *et al.*, 1966; Aghajanian & Roth, 1970).

A number of recent *in vivo* observations suggest that GHB may increase brain DA, in part by enhancing its binding or preventing its release from dopaminergic neurones (Aghajanian & Roth, 1970; Roth & Surh, 1970; Roth, 1971; Roth, 1972; Walters & Roth, 1972). In order to study this further, it was important to develop a system that allowed a more direct approach to this problem.

It has been observed that labelled DA accumulated by striatal slices and DA formed from labelled precursors may be liberated by electrical field stimulation (Baldessarini & Kopin, 1966; Ng, Chase, Colburn & Kopin, 1971). Preliminary experiments in this laboratory indicate that K^+ ions may also be used as a simple investigative procedure to induce and study the release of labelled DA from striatal slices (Bustos & Roth, 1972). The purpose of this paper is two fold: (1) to examine further the K^+-induced release of DA and the ionic requirements for this release, and (2) to study the effect of GHB on spontaneous and K^+-induced release of DA from the striatum. For the sake of comparison the effect of GHB on release of 5-hydroxytryptamine (5-HT) from the striatum and noradrenaline (NA) from the hypothalamus was also investigated.

Methods

Release studies utilizing a lucite chamber

Male Sprague-Dawley rats, weighing about 200–250 g, were decapitated and the left and right striata were rapidly dissected. Striatal tissue slices (0·18 mm in thickness) were prepared with a Sorvall tissue chopper. Slices weighing about 70 mg, were incubated for 30 min at 37° in 5·0 ml of Krebs-Ringer-phosphate (KRP), pH 7·4, saturated with 95% O_2 + 5% CO_2 and containing either ^{3}H-dopamine (3,4-dihydroxyphenylethyl-1-^{3}H(N)-amine; specific activity 8·3 Ci/mM; final concentration, 1×10^{-7}M), L-tyrosine-3,5-^{3}H (specific activity 25 Ci/mM; final concentration of 4×10^{-8}M) or L-tryptophan-^{3}H (specific activity 5·3 Ci/mM; final concentration of 4×10^{-7}M).

The release of ^{3}H-DA previously taken up by slices was examined as follows: at the end of the incubation period, the slices were transferred to a 5·0 ml lucite chamber with a nylon mesh bottom (pore size=35 microns) which permitted a complete and rapid (15 seconds) separation between slices and medium, while maintaining slices inside the chamber. The striatal slices were washed for a period of two minutes with KRP and the release of ^{3}H-DA was examined by transferring the lucite chambers containing the tissues through successive beakers containing 5·0 ml of normal KRP or KRP modified according to the experimental situation. The spontaneous release of radioactivity into the medium was followed for a total period of 35 min (5 min in each of 7 beakers) before nerve cell depolarization induced release of ^{3}H-DA was produced by K^+ (53·0 mM) for a period of 3·0 to 5·0 minutes.

The release into the medium of ^{3}H-DA and ^{3}H-5-HT formed respectively from ^{3}H-tyrosine and ^{3}H-tryptophan was followed in general as described above. In this case, however, spontaneous release of radioactivity was followed for two consecutive periods of 3·0 min each, before stimulation with K^+.

Carrier DA or 5-HT (50 μg of each) was added to the medium and separation and analysis of the media for labelled DA and 5-HT was carried out by column chromatography (for details see below). Unless stated otherwise, release was expressed as cpm of ^{3}H-DA or 5-HT released from 100 mg of striatum into 5·0 ml of incubation medium/3·0 minutes. For simplification, in the text and under the figures this is stated as (cpm of ^{3}H-DA or ^{3}H-5-HT released)/100 mg striatum.

Release studies with a superfusion system

In this group of experiments the release of labelled monoamines was followed from striatal slices as well as from slices obtained from the hypothalamus. The hypothalamus was dissected from rat brain as described by Glowinski & Iversen (1966). Hypothalamic slices of 0·17 mm in thickness were prepared with a Sorvall tissue chopper. Ten mg of striatal or hypothalamic slices were incubated for 30 min at 37° C in 2·0 ml KRP, pH 7·4, saturated with 95% O_2+5% CO_2 and containing either ^{3}H-tyrosine (2×10^{-7}M) or ^{3}H-DA (3×10^{-7}M). The slices were then transferred to superfusion chambers (2·0 ml capacity) from which the release of exogenously taken-up ^{3}H-DA or newly synthesized ^{3}H-DA or ^{3}H-NA was followed. The superfusion chamber was suspended in a moist, funnel-shaped chamber, maintained at 37±0·5° C by a heated water jacket. The slices were washed with 5·0 ml of KRP and then superfused with KRP solution which was being continuously oxygenated and pre-warmed to 37° C. A roller pump (extracorporeal) was used to maintain a constant flow of 4 ml/min, and a system of three-way taps enabled selection of fluids from different reservoirs to be made without disturbing the flow. Stimulation of release was carried out for 1·0 min by switching the superfusion to KRP containing high K^+ (53·0 mM). When release from striatal slices of exogenously loaded ^{3}H-DA was to be studied, an initial superfusion period of 15·0 min was allowed before release was stimulated with K^+. This procedure allowed for a constant and steady basal release prior to stimulation. Potassium-induced release from striatal or hypothalamic slices of newly formed ^{3}H-DA or ^{3}H-NA respectively, was started after 10 min of superfusion with normal KRP. Samples containing the released material were collected every min into tubes containing 1·0 ml of trichloracetic acid (TCA) 50% and carrier DA and NA (50 μg of each). Catechols were extracted from the tissues with 15% TCA. All the samples were immediately frozen and kept for chromatographic analysis.

The superfusion chambers described above allowed a complete recovery of the slices at the end of the superfusion period. Release of radioactive DA or NA was expressed then as percentage of total ^{3}H-DA or ^{3}H-NA found in the tissue and in different collecting tubes at the end of the superfusion period.

Column chromatography

Alumina columns were used to concentrate the catecholamines and to separate them from tyrosine, *O*-methylated catecholamine metabolites and tritiated water (Roth & Stone, 1968; Boadle-Biber, Hughes & Roth, 1970). The alumina (British Drug Houses, Ltd.) was washed repeatedly to remove fines and then poured

to give columns 1·5 × 0·4 cm in size. Tris buffer (0·2 ml, 1 M, pH 8·2) and disodium edetate (final concentration, 10 mg/ml) were added to the samples and the pH adjusted to 8·4 with NaOH. The samples were passed through the columns immediately and recycled once; the column was then washed with 40·0 ml of distilled water, to remove residual tyrosine, and blown dry. Catecholamine and deaminated metabolites were eluted by suspending the alumina in 2·0 ml of 0·2 M perchloric acid, draining and then washing with 2·0 ml of distilled water. The recovery of NA and DA by this procedure was between 81 and 83 per cent. Reported values are not corrected for recovery.

Long Amberlite CG-120 columns (14 × 0·4 cm) were used to separate DA and NA from their deaminated metabolites and from 3-hydroxytyrosine (DOPA) in the perchloric acid eluates from the alumina columns (Stjärne & Lishajko, 1966; Roth & Stone, 1968).

Analysis of labelled 5-HT released into the medium was carried out by ion exchange chromatography through Dowex 50 × 4 (Na^+) columns (2·5 × 0·3 cm in size) prepared according to Häggendal (1963). The columns were first washed with 5·0 ml of acetate buffer, 0·1 M, pH 6·0. Then the samples were acidified with HCl and passed through the columns which were rinsed further with 25·0 ml of acetate buffer. This last procedure removed the tritiated tryptophan. The 5-HT was then eluted with 4·0 ml of Na_3PO_4, 0·6 M (Costa, Spano, Grapetti, Algeri & Neff, 1968). Recovery of 5-HT by this method was about 80% and reported values are not corrected for recovery.

Scintillation spectrometry

Samples and eluates from the columns containing labelled DA, NA and 5-HT were analysed for tritium in a Packard Scintillation Counter (model 3375) to a constant standard deviation of 1·5%. The samples were counted in 20·0 ml of dioxane-ethanol-toluene solvent (1 litre each of toluene, dioxane and ethanol containing 240 g naphthalene, 15 g 2,5-diphenyloxazole and 0·3 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene.

Solutions

The Krebs-Ringer-phosphate used in these experiments had the following composition: NaCl 128 mM; KCl 4·8 mM; $CaCl_2$ 0·75 mM; $MgSO_4$ 1·20 mM; glucose 16 mM; Na_2HPO_4 16 mM at pH 7·4; sodium ascorbate, 20 mg/litre.

Krebs-Ringer-phosphate with high K^+ (Hyperosmolar KRP-high K^+) was usually made by increasing the concentration of KCl to 53·0 mM without altering the concentration of NaCl. In some experiments Krebs-Ringer-phosphate-high K^+ was made by replacing proportions of NaCl with equimolar amounts of KCl (Isosmolar-KRP-high K^+). Other modifications of the KRP are described in the text.

Drugs and chemicals

L-Tyrosine-3,5-^{3}H, ^{3}H-dopamine, L-tryptophan-^{3}H and ^{14}C-urea (specific activity 57 mCi/mmol) were obtained from New England Nuclear Corp., Mass. Wyett Laboratories, Inc., Pa., provided the 4-OH butyrate sodium salt (GHB) used in this work.

Results

Spontaneous and K^+-induced release of biogenic amines from the striatum as studied with lucite chamber

Effect of K^+ on ^3H efflux. Figure 1 shows the typical efflux pattern of ^3H from striatal slices initially loaded with ^3H-DA. A slow and almost constant release of label was observed during the initial 35·0 min in which the tissues were transferred through beakers containing normal KRP. Addition of K^+ (53·0 mM) for 3·0 min to the medium provoked a fast and large increase in the efflux of ^3H. Return of the lucite chambers to beakers with normal KRP was paralleled with restoration of the basal release. Analysis through alumina columns showed that almost 50% of the increase in ^3H-efflux after K^+ stimulation was accounted for by ^3H-catechols (DA + deaminated catechols). Thirty-five per cent of the ^3H-efflux prior to K^+ stimulation was ^3H-catechols. Further analysis through Amberlite columns showed that ^3H-DA accounted for 80–85% of the ^3H-catechols in either condition.

Effect of K^+, Ca^{++} and Mg^{++} ions on DA release. Potassium stimulation (53·0 mM) for 3·0 min produced an almost 20-fold increase in the release of ^3H-DA previously taken up into striatal tissues (Fig. 2). Surprisingly, this K^+-induced release was not altered when it took place in Ca^{++}-free KRP media fortified with the disodium salt of ethylene diamine tetra acetic acid (EDTA) (3×10^{-4}M). However, the addition of $MgCl_2$ (final concentration, 12·0 mM) to the Ca^{++}-free KRP produced a complete block in the K^+-induced release of exogenously loaded ^3H-DA.

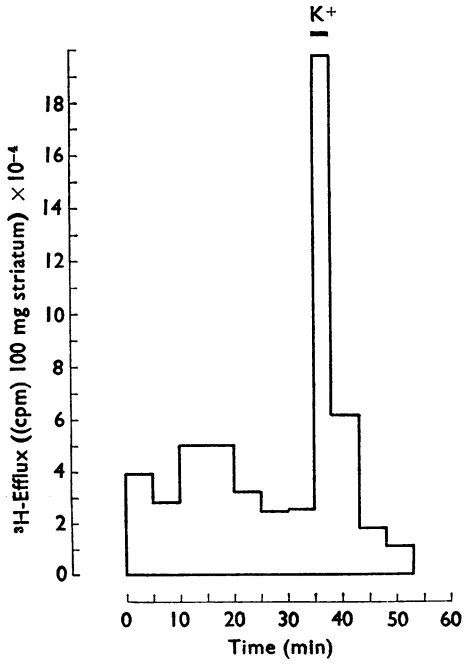

FIG. 1. Typical pattern of spontaneous and K^+-induced efflux of ^3H from striatal slices loaded with ^3H-DA. Striatal slices were incubated in normal KRP for 30 min at 37° C and then transferred to lucite chambers in which release was measured as described in the text. K^+ stimulation (53·0 mM) is shown by the dark line at the top of the figure.

Potassium (53·0 mM) also produced a 27-fold increase in the release of ^{3}H-DA newly synthesized from ^{3}H-tyrosine (Table 1). In this particular experiment the spontaneous release of ^{3}H-DA was followed for a period of 35·0 min prior to K^+ stimulation. Absence of Ca^{++} and presence of EDTA in the KRP also produced no inhibition in the K^+-evoked release of newly formed ^{3}H-DA (Table 1) under these conditions. The spontaneous release of ^{3}H-DA newly synthesized or previously taken up into tissues was also not altered when release was followed in KRP media free of $CaCl_2$.

Effect of γ-hydroxybutyrate on release of ^{3}H-dopamine and ^{3}H-5-hydroxytryptamine from the striatum

Table 2 shows the effect of GHB on K^+-induced release of newly formed ^{3}H-DA and ^{3}H-5-HT and of exogenously taken up ^{3}H-DA. In this experiment the striatal slices were incubated in the presence of the tracer as described under **Methods** and the spontaneous and K^+-induced release was followed both in presence and absence of the drug. The addition of GHB (1×10^{-3}M) to the lucite chambers produced a significant ($P<0·01$) 30% inhibition of the K^+-evoked release of newly formed ^{3}H-DA. γ-Hydroxybutyrate at a concentration of 5×10^{-4}M produced no inhibitory effect. The drug, at either concentration, had no effect on the spontaneous release of newly formed ^{3}H-DA.

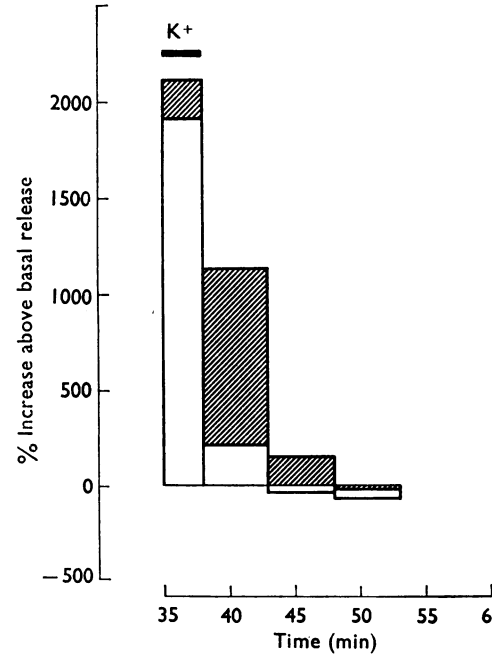

FIG. 2. Effect of Ca^{++} on K^+-induced release of exogenous ^{3}H-DA from the striatum. Striatal slices were incubated in normal KRP for 30 min at 37° C in the presence of ^{3}H-DA, as described in the text, and then transferred to lucite chambers. Release of ^{3}H-DA was then measured in either normal KRP (open columns) or Ca^{++}-free KRP plus EDTA (cross-hatched columns). Stimulation by K^+ (53·0 mM), after following spontaneous release for 35 min, is shown by the dark line at the top of figure. Basal release of ^{3}H-DA before K^+ stimulation was 2,380 and 3,600 (cpm)/100 mg striatum respectively when followed in normal KRP and Ca^{++}-free KRP. This figure represents the mean of two experiments.

In contrast with the release of newly formed DA, the K^+-induced release of labelled DA from tissues previously loaded with ^{3}H-DA was unaltered by GHB (Table 2). Under these experimental conditions, however, K^+ stimulation produced a more than 18-fold increase in release. Spontaneous release before stimulation was of the order of 3,600 (cpm)/100 mg striatum.

The K^+-induced release of labelled 5-HT newly formed from ^{3}H-tryptophan was also followed in the presence of GHB. Again, in contrast to tissues loaded with tyrosine, GHB was found to be without effect on the release of newly formed 5-HT.

Spontaneous and K^+-induced release of biogenic amines studied with a superfusion system

Effect of repeated K^+ stimulation on release of newly formed dopamine from the striatum. It seemed possible that the rather selective effect of GHB on release of newly synthesized ^{3}H-DA could actually be due to a more rapid reuptake by the tissue of the newly formed ^{3}H-DA in the presence of the drug. Moreover, the lack of effect of Ca^{++}-free KRP on K^+-induced release of ^{3}H-DA reported in the last section was puzzling. We changed therefore to a system that allowed a faster washout of label from the tissues than the previous system.

TABLE 1. *Effect of Ca^{++} on K^+-induced release of newly synthesized ^{3}H-dopamine from striatum as measured in lucite chamber*

	Release of ^{3}H-DA ((cpm)/100 mg striatum)	
	Release before K^+ stimulation	Release during K^+ stimulation
Normal KRP	349, 315	10674, 7874
Ca^{++} free KRP plus EDTA (3×10^{-4} M)	554, 350	10028, 8432

Striatal slices were incubated in normal KRP for 30 min at 37° C in the presence of ^{3}H-tyrosine, as described in the text, and then transferred to lucite chambers. Release of ^{3}H-DA was then measured in either normal KRP or Ca^{++} free KRP plus EDTA. Slices were exposed to K^+ (53 mM) for 3 min after following basal release for 35 minutes. Values for two experiments are presented.

TABLE 2. *Effect of γ-hydroxybutyrate on K^+-induced release of ^{3}H-dopamine and ^{3}H-5-hydroxytryptamine*

Precursor	Net release of ^{3}H-DA ((cpm)/100 mg striatum)	Net release of ^{3}H-DA in presence of GHB (1×10^{-3} M) ((cpm)/100 mg striatum)	% Change after GHB	Significance
^{3}H-Tyrosine	9,620±1,780 (4)	7,260±1,790 (4)	−27·3± 6·1	$P<0.01$
^{3}H-Dopamine	60,700±3,422 (4)	65,000±6,360 (4)	+8·8±15·4	N.S.
	Net release of ^{3}H-5-HT ((cpm)/100 mg striatum)	Net release of ^{3}H-5-HT in presence of GHB (1×10^{-3} M) ((cpm)/100 mg striatum)		
^{3}H-Tryptophan	805±219 (3)	815±218 (3)	+0·9±4·7	N.S.

Striatal slices were incubated in normal KRP for 30 min at 37° C in the presence of radioactive precursors. Release was then measured in lucite chambers, as described in the text, in the presence or absence of GHB (1×10^{-3} M). Net release represents the total cpm of ^{3}H-DA or ^{3}H-5-HT being released in the media by exposure to high K^+ (53·0 mM) for a period of 3·0 min, minus the spontaneous release occurring in the period immediately prior to K^+ stimulation. Values shown represent the mean±S.E. Numbers in parentheses indicate number of individual experiments. Statistical significance was calculated by Student's *t* test for paired data.

In this experiment, 10·0 mg of striatal slices were incubated in normal KRP in the presence of ^{3}H-tyrosine, washed and then transferred to superfusion chambers in which the release of newly formed ^{3}H-DA was to be followed. It can be seen that an exposure of only 1·0 min to hyperosmotic KRP containing 53·0 mM K^+ produced a marked increase in the release of newly synthesized ^{3}H-DA (Fig. 3). On changing to a medium with normal K^+, there was a rapid return of DA release to basal levels. A second K^+ stimulation produced a similar response and recovery after changing to normal KRP (Fig. 3).

Striatal slices incubated with ^{14}C-urea showed no increased release of labelled urea after K^+ stimulation under similar experimental conditions (Fig. 4).

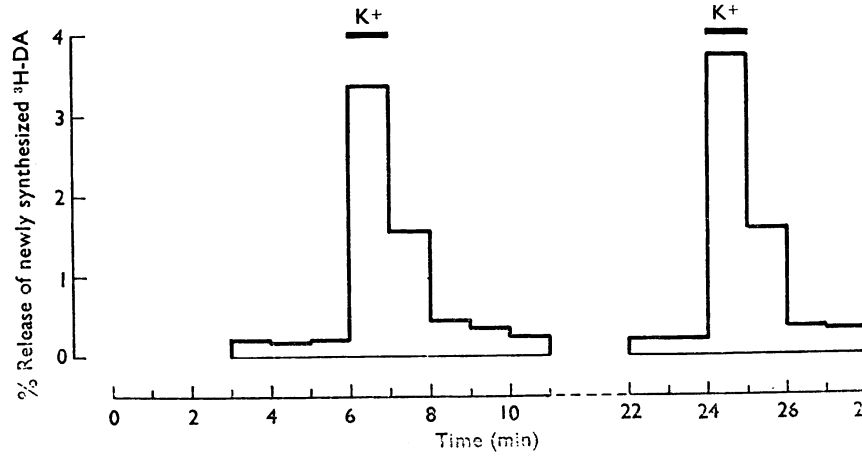

FIG. 3. Effect of repeated K^+ stimulation on release of newly formed ^{3}H-DA. Striatal slices were incubated for 30 min at 37° C in the presence of ^{3}H-tyrosine, as described in the text, and then transferred to the superfusion system to measure release. Stimulation by K^+ (53·0 mM) is shown by dark lines at the top of the figure. Total ^{3}H-DA synthesized amounts to 110,700 cpm ± 20,700 (mean ± S.E. of three experiments).

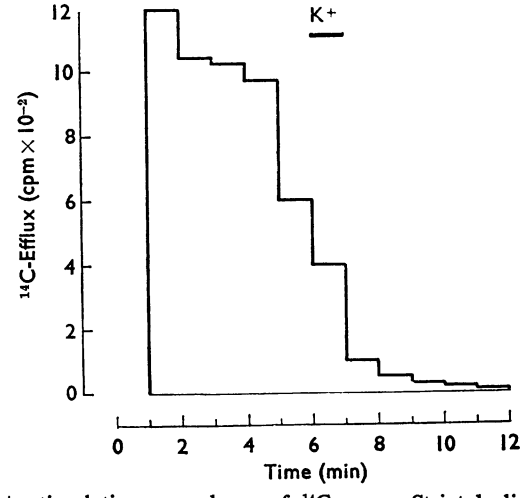

FIG. 4. Effect of K^+ stimulation on release of ^{14}C-urea. Striatal slices were incubated in KRP for 30 min at 37° C in the presence of 10 μCi of ^{14}C-urea and then release was measured in the superfusion system. The 1 min K^+ stimulation is shown by dark line at top of figure.

Effect of Ca^{++} on release of newly formed dopamine from the striatum

In this experiment striatal slices were incubated in normal KRP in the presence of ^{3}H-tyrosine, washed and then maintained for 5·0 min in Ca^{++}-free KRP before starting the superfusion with this last medium. The omission of Ca^{++} from the KRP produced an almost 90% inhibition of the release of newly formed ^{3}H-DA when changing to hyperosmotic KRP-high K^+ for 1·0 min (Fig. 5). For comparison the release after high K^+ in presence of normal Ca^{++} concentrations has been included. Lack of Ca^{++} also produced similar inhibitory effects on release when the ^{3}H-DA release was induced by an isoosmotic KRP-high K^+ medium (that is replacing the NaCl by equimolar amounts of KCl).

Effect of Ca^{++} on release of exogenously loaded ^{3}H-dopamine

The K^+-induced release of ^{3}H-DA previously taken up into striatal slices was shown to be highly dependent on the presence of Ca^{++} in the medium (Table 3) when analysed by the superfusion technique. Potassium produced an almost 30-fold increase in the release of label when it was followed in normal KRP. However, the absence of Ca^{++} completely abolished the K^+-induced release of exogenously loaded ^{3}H-DA. No difference was found whether K^+ stimulation was carried out under hyperosmotic or isoosmotic conditions.

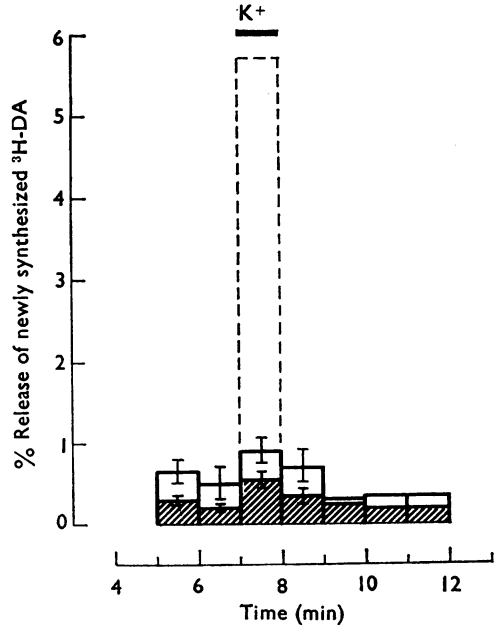

FIG. 5. Effect of Ca^{++} on release of newly synthesized ^{3}H-DA from the striatum as measured by the superfusion system. Incubation conditions were essentially as described in the text. Spontaneous and K^+-induced release was followed in KRP Ca^{++}-free+EDTA (3×10^{-4}M) medium with (cross-hatched columns) and without GHB (1×10^{-3}M (open columns). Vertical bars represent the standard error of the mean. Total ^{3}H-DA synthesized in these tissues amounts respectively to 141,164 and 121,769 cpm. For comparison, the ^{3}H-DA release after high K^+ in normal Ca^{++} medium is shown (vertical dashed column).

Effect of γ-hydroxybutyrate on K⁺-induced release of newly synthesized ³H-dopamine

In these experiments, striatal slices were incubated in normal KRP plus ³H-tyrosine, washed and then maintained for 5·0 min in KRP with or without GHB (1×10^{-3}M) before starting the superfusion. Figure 6 shows that the presence of GHB in the superfusion medium was able to antagonize significantly ($P<0{\cdot}05$, $n=3$) the K^+-evoked release of newly synthesized ³H-DA. Potassium-induced release in this experiment was carried out by using a hypersomotic KRP-high K^+

TABLE 3. *Effect of Ca^{++} on K^+-induced release of exogenous ³H-dopamine from striatum as measured by the superfusion system*

	Release of ³H-DA (% of ³H-DA taken up by tissues)	
	Release 1 min before K^+ stimulation	Release during K^+ stimulation
Normal KRP	0·50, 0·62	17·30, 15·00
Ca^{++}-free KRP plus EDTA (3×10^{-4}M)	0·44, 0·38	1·70, 1·52

Striatal slices were incubated in normal KRP for 30 min in the presence of ³H-DA, as described in the text, and then transferred to the superfusion system. Release of ³H-DA from the tissues was then measured in either normal KRP or Ca^{++}-free KRP. The tissues had taken up an average of 285,037 cpm and 310,664 cpm of ³H-DA respectively. Potassium stimulation was carried out for 1·0 minute. Values for two experiments are presented.

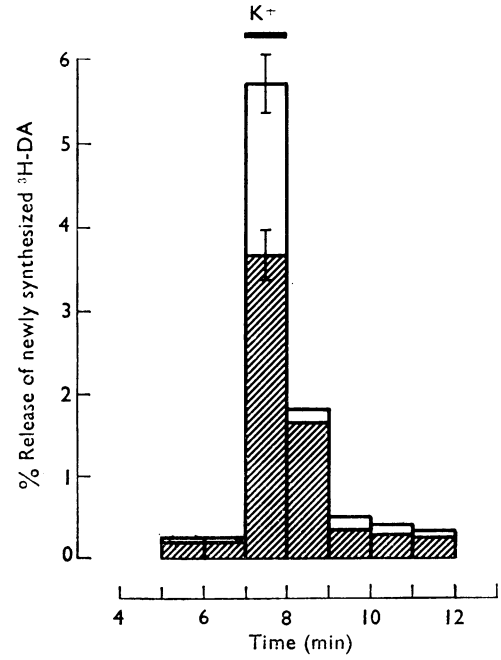

FIG. 6. Effect of GHB (1×10^{-3}M) on release of newly synthesized ³H-DA from the striatum as measured by the superfusion system. Striatal slices were incubated with KRP containing ³H-tyrosine as described in the text. Spontaneous and K^+-induced release from the tissues was then followed in KRP with (cross-hatched columns) and without GHB (open columns). Vertical bars represent the standard error of the mean. Total ³H-DA synthesized in these tissues amounts respectively to 136,592±19,161 and 100,038 cpm±14,200 (mean±S.E.). Release of ³H-DA by K^+ in the presence of GHB is significantly different from the control ($P<0{\cdot}05$, $n=3$).

medium. When the evoked release of DA was studied under isoosmotic conditions (see **Methods** for composition of the medium) GHB was also found to produce a 37% inhibitory effect on this release ($P<0.05$, $n=5$). No effect of GHB was found on spontaneous release of newly formed ^{3}H-DA in either medium.

The effect of GHB on K^+-evoked release of newly formed ^{3}H-DA followed in a Ca^{++}-free KRP medium is shown in Figure 5. The drug was found to have no further effect on release under these experimental conditions.

Effect of γ-hydroxybutyrate on release of newly synthesized ^{3}H-noradrenaline from the hypothalamus

The next experiment showed that GHB not only affects the release of newly formed monoamines from the striatum but also from the hypothalamus. The presence of GHB (1×10^{-3}M) in the superfusion medium was found to inhibit significantly the K^+-induced release of newly synthesized ^{3}H-NA from hypothalamic slices ($P<0.05$, $n=4$) (Table 4). Spontaneous release of newly synthesized ^{3}H-NA was very small under the experimental conditions used.

Discussion

The results described in this paper indicate that it is possible to use short exposures to K^+ as a simple experimental tool to evoke release from the striatum of either labelled DA previously taken up or previously synthesized from labelled precursors. The effect of K^+ appears to be somewhat specific since it did not influence the release of ^{14}C-urea from striatal slices. Moreover, the integrity of the tissue was demonstrated by the fact that repeated K^+ stimulation of release of newly formed ^{3}H-DA showed similar responses and recovery, when the superfusion system was employed.

Calcium ions seem to be a necessary factor for the release of certain hormones and many putative neurotransmitters. Release of NA from the adrenal medulla (Douglas, 1966), peripheral sympathetic nerve (Burn & Gibbons, 1964; Boullin, 1967), atria (Katz & Kopin, 1969a) and brain slices (Baldessarini & Kopin, 1967; Katz & Kopin, 1969b) is calcium dependent. Similar Ca^{++} dependence has been shown for the electrically evoked release of vasopressin from the isolated neurohypophysis (Douglas & Poisner, 1964). The results obtained in this work with the superfusion system indicate that K^+ depolarization of striatal slices induces a release of DA which is also highly dependent on the presence of Ca^{++} in the

TABLE 4. *Effect of γ-hydroxybutyrate on K^+-induced release of newly synthesized ^{3}H-noradrenaline from the hypothalamus*

	K^+-induced release of ^{3}H-NA (% of newly synthesized ^{3}H-NA)
KRP normal	7·93±0·41
KRP+GHB (1×10^{-3}M)	5·55±0·34 *

Hypothalamic slices were incubated with KRP and ^{3}H-tyrosine as described in the text. Spontaneous and K^+-induced release from the tissues was followed in the superfusion system in either the absence or presence of GHB. The tissues exposed to KRP or KRP+GHB had previously synthesized respectively 7,159±337 and 7,875±1,200 cpm of ^{3}H-NA (mean±S.E.). Values in the table represent the ^{3}H-NA released during and 1·0 min after K^+ stimulation (mean±S.E.). * $P<0.05$ ($n=4$). Student's t test for paired data.

medium. This dependence on Ca^{++} was present both when the DA released was newly synthesized amine or was previously taken up by slices. The dependence of K^+-evoked release on Ca^{++} remained the same whether it was studied in hyperosmolar KRP-high K^+ with normal Na^+ concentrations or isoosmolar KRP-high K^+ with low Na^+ concentrations. Similarly the induced release studied under normal Ca^{++} concentrations did not differ whether it was provoked under hyper- or iso-osmolar conditions. These results are not surprising since the tissues were exposed to these media for only 1·0 min; that is only during the time of K^+ stimulation. These controls were necessary, however, since the stimulation by K^+ involved the changing of the superfusion from a medium of defined ionic composition to one of altered composition due to the presence of high K^+.

One surprising finding was the fact that it was not possible to show that release was Ca^{++} dependent when the lucite chamber was used to measure it. However, high Mg^{++} concentrations (12·0 mM) did inhibit the release. If Ca^{++} is tightly bound to the striatal tissue it is possible that release studied in the lucite chamber did not allow a complete washout of Ca^{++} present in the tissue. Enough Ca^{++} would be present then to permit the K^+-evoked release. However, measurement of Ca^{++} concentrations and Ca^{++} efflux from striatal slices would be necessary to support this view. Using a system similar to our lucite chamber in order to measure ^{45}Ca efflux from brain cortical slices, Cooke & Robinson (1971) have shown that 10% of the initial ^{45}Ca was still present at the end of 35 min of efflux. This is the time of equilibration chosen in our lucite system before evoking release of DA with K^+.

γ-Hydroxybutyrate at a concentration of 1 mM was shown to have an inhibitory effect on the K^+-induced release of newly formed ^3H-DA from the striatum. This effect was shown in both the relatively slow transfer lucite system and the superfusion system. The possibility remains that this effect of GHB on release could actually be due to a more rapid reuptake by the dopaminergic neurones of the newly formed DA. This is unlikely, however, for several reasons: (1) this effect was shown even after K^+ stimulation of 1·0 min during which the reuptake of ^3H-DA should be very low; (2) it was shown in the presence of high K^+ concentrations, which probably are inhibitory to the active reuptake mechanism of DA (Bogdanski, Tissari & Brodie, 1968; Bogdanski & Brodie, 1969; Harris, 1972); and (3) experiments in our laboratory have indicated that GHB (1×10^{-3}M) has no effect on the active uptake of ^3H-DA by striatal synaptosomal preparations either in *in vitro* conditions or from animals pretreated with anaesthetic doses of GHB (Bustos, Kuhar & Roth, in preparation). The effect of GHB on release of newly formed DA might explain, in part, why this agent causes a rapid increase in striatal levels of DA when the drug is administered *in vivo*. The GHB concentration found to be effective in these *in vitro* studies is similar to that found in the brain after administration of anaesthetic doses of GHB at a time when the DA levels in the striatum begin to increase (Walters & Roth, 1971). Other concentrations of GHB tested in this work (5×10^{-4}M) had no effect *in vitro* in agreement with *in vivo* results (Giarman & Roth, 1964).

In contrast with the inhibitory effect of GHB on evoked release of newly synthesized ^3H-DA this drug did not affect the K^+-induced release of ^3H-DA taken up by the tissue. There are at least two explanations for this finding. First, the mechanism of release of ^3H-DA synthesized from tyrosine may differ from the

release of ^{3}H-DA previously taken up into striatal slices. However, at present there is no sound experimental evidence to support this. Second, dopaminergic terminals may contain a DA compartment from which the newly synthesized amine is preferentially released or utilized. Recent studies by Thierry, Blanc & Glowinski (1971) on the disposition of catecholamines from central noradrenergic terminals of the rat have indicated that NA newly taken up or newly synthesized is mainly localized in a small compartment with a half-life between 15–30 minutes. The amine seems to be preferentially released from this compartment which has been regarded as a 'functional compartment'. Exogenous or endogenously synthesized amines stored for a longer period of time seem to be localized in a larger compartment with a half-life between 180–260 minutes. This last compartment has been assumed to be a 'storage compartment' in equilibrium with the 'functional compartment'. It is possible then that newly synthesized ^{3}H-DA readily goes into this 'functional compartment' which is probably located close to the release sites at the presynaptic membrane. Exogenous ^{3}H-DA probably not only goes to this compartment but also to the 'storage compartment'. Regarding this point, it is worth mentioning that K^+ stimulation for 1·0 min in our superfusion system released 5% of the newly synthesized ^{3}H-DA as compared to a 15% release of the exogenously taken up ^{3}H-DA. If GHB only interacts with the 'functional compartment' this might explain why the inhibitory effect of this drug is not clearly seen when evoked release of exogenous ^{3}H-DA is studied. The effect of GHB on release from the functional compartment would be in a way diluted by the presence of ^{3}H-DA in the 'large' or 'storage' compartment.

Our experiments, however, do not offer any adequate explanation for the mechanisms that are responsible for the inhibitory effect of GHB on release of ^{3}H-DA. This effect could be located at any of the stages associated with the release of the neurotransmitter, that is: influx of Ca^{++}; interaction between Ca^{++} and free or bound form of neurotransmitters; interaction with membrane (either synaptic vesicles or presynaptic membranes) and thereby slowing the process of release. One clue to the mechanism involved is the fact that the effect of GHB was not seen when evoked release of ^{3}H-DA was studied in the absence of Ca^{++}. Although release of ^{3}H-DA under these circumstances was very low even in these conditions K^+ stimulation was able to produce a two-fold increase in release. However, further studies are necessary to see whether GHB exerts its effects through Ca^{++} ions. It is interesting that an analogue of GHB, butyrate, has been shown to interact with membrane model receptors and to form molecular complexes with dopamine and other monoamines (Rizzoli & Galzigna, 1970). Whether this is also the case for GHB remains to be shown.

The observation that GHB blocked the release of newly synthesized DA but not newly formed 5-HT is in agreement with the *in vivo* findings in which no striking increase in the brain level of 5-HT was found after GHB (Gessa *et al.*, 1966; Roth & Surh, 1970). It should be mentioned that the absence of Ca^{++} does not appear to interfere with the evoked release of ^{3}H-5-HT from brain slices (Chase, Katz & Kopin, 1969; Katz & Kopin, 1969b). Thus it appears that the mechanism of release of 5-HT from central neurones may differ somewhat from that of DA. If Ca^{++} is a necessary element for the action of GHB, this might partly explain why this drug has a selective action on striatal dopaminergic neurones and not on serotoninergic ones.

Another interesting observation was the fact that the inhibitory effect of GHB on K^+-evoked release of monoamines does not seem to be restricted to the striatum but also affects other regions of the brain such as the hypothalamus. Why is it then that an increase of DA in the striatum is found but no change of NA concentration in the hypothalamus after the drug? One possibility is that the turnover of NA in hypothalamic neurones is much slower than the turnover of DA in the striatum. Alternatively, the contribution of the release to the overall turnover of the neurotransmitter is higher in striatal dopaminergic neurones than in hypothalamic noradrenergic neurones. Iversen & Glowinski (1966) showed that the rat brain region with the most rapid apparent ^{3}H-NA turnover is the cerebellum and that with the slowest is the hypothalamus. Similar results have been reported by Chalmers & Wurtman in the rabbit (1971). Another consideration is the possibility that factors which regulate synthesis, uptake and release of neurotransmitters in the striatum differ from those of the hypothalamus. These possibilities are currently under investigation. Finally, it may be that this direct effect of GHB on release plays only a minor role *in vivo* to account for the increase in brain DA after parenteral administration of this drug. Evidence is accumulating which appears to indicate that at least a portion of GHB's action is exerted at the level of the DA containing cell bodies located in the zona compacta of the substantia nigra (cf. Roth, 1972). GHB or γ-butyrolactone administered in anaesthetic doses cause abolition in the firing of these DA containing neurones which is probably responsible, at least in part, for the marked decrease in the amount of transmitter released from the nerve terminals of this neuronal system (Walters, Aghajanian & Roth, in preparation). At the present time this appears to be the most likely explanation for the rapid increase in DA levels observed after GHB administration. However, other mechanisms such as a direct effect of GHB on DA containing nerve terminals cannot be completely ruled out.

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