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EFFECT OF CARBONYL-BINDING AGENTS AND OXIDATIVE PHOSPHORYLATION UNCOUPLERS ON THE RELEASE OF [^{3}H]NOREPINEPHRINE FROM MOUSE HEART

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Abstract Three carbonyl reagents, namely sodium bisulfite, hydroxylamine and phenylhydrazine, increased the release of [^{3}H]norepinephrine from sliced mouse heart during stimulation with 50 mM potassium ion. The [^{3}H]norepinephrine was identified by binding to aluminum hydroxide and subsequent thin-layer chromatography. These carbonyl reagents at 10^{-3} M increased the overflow of [^{3}H]norepinephrine by 162–249 per cent. Semicarbazide, another carbonyl reagent, proved to be an exception. However, semicarbazide reacted more slowly than the other carbonyl reagents with pyridoxal phosphate (which was used as a model aldehyde). Therefore, the ability of carbonyl reagents to increase overflow of [^{3}H]norepinephrine appeared to correlate with their ability to react with carbonyl groups. The augmented release of [^{3}H]norepinephrine by sodium bisulfite, hydroxylamine or phenylhydrazine was not due to inhibited reuptake of released transmitter since cocaine, a strong uptake blocker, did not increase the release of [^{3}H]norepinephrine. When animals were pretreated with 6-hydroxydopamine to destroy adrenergic nerve terminals, the stimulus-induced overflow of [^{3}H]neurotransmitter in the presence of sodium bisulfite was virtually abolished. Similar results were obtained when cocaine was used to prevent the accumulation of [^{3}H]norepinephrine by nerves. Thus, the adrenergic nerve endings were the source of the increased overflow of [^{3}H]norepinephrine. These data implicate endogenous aldehydes or ketones in a regulatory role during the release of neurotransmitter. Experiments with norepinephrine-depleted hearts (reserpine, 16 hr) labeled with [^{3}H]metaraminol (not metabolized by monoamine oxidase) as neurotransmitter showed increased release of ^{3}H-neurotransmitter in the presence of 10^{-3} M sodium bisulfite. This indicated that the aldehyde derived from norepinephrine by the action of monoamine oxidase was not responsible for the action of the carbonyl-binding agents. In separate experiments, two oxidative phosphorylation uncouplers, 2,4-dinitrophenol (5×10^{-4} M) and carbonyl cyanide *m*-chlorophenylhydrazone (10^{-6} M), similarly increased overflow of [^{3}H]norepinephrine. Experiments with cocaine showed that the increased release of [^{3}H]norepinephrine in the presence of carbonyl cyanide *m*-chlorophenylhydrazone was derived from nerve terminals. The oxidative phosphorylation uncouplers and the carbonyl reagents may have a common site of action, perhaps at an adenosine triphosphate-dependent carbonyl site in the axonal membrane.

The release of norepinephrine (NE) has been studied with isolated organs or tissue slices that were exposed to either potassium [1, 2] or electrical stimulation [3, 4]. The amount of NE released from stimulated sympathetic nerve terminals is influenced by a number of factors. These factors include activation of cholinergic receptors [5], blockade of α-receptors [3, 6] and stimulation of α-receptors [7]. Additionally, prostaglandins regulate the release of NE, apparently by influencing the influx of calcium ions into the neuron [8]. The data dealing with α-receptor blockade or stimulation have led some investigators [3, 4, 6, 7, 9] to hypothesize that the regulation of transmitter release was via a feedback mechanism involving either pre- or postsynaptic receptors or both. We were interested in exploring the possibility that 3,4-dihydroxyphenylglycolaldehyde (the aldehyde derived from NE by the action of monoamine oxidase) might play a role in negative feedback regulation of NE release. Therefore, aldehyde-binding agents were tested for their effect on the K^+-stimulated release of NE from mouse heart. Although sodium bisulfite, hydroxylamine and phenylhydrazine did, in fact, increase the overflow of NE, the mechanism of action did not appear to be the one postulated.

MATERIALS AND METHODS

Male, Swiss–Webster mice (25–30 g) were killed by cervical dislocation. Hearts were removed and sliced open longitudinally from the apex so that the two halves remained joined near the atria. Each heart was rinsed briefly in cold 0.9% (w/v) saline and then spread out on cold moist (0.9% saline) filter paper on the stage of a McIlwain-Mickle tissue chopper. The heart was chopped from the apex of one half to the apex of the other at a setting of 0.5 mm. Each heart was put into a 10-ml beaker containing 2 ml of cold, modified Krebs–Ringer phosphate buffer and equilibrated for 5 min at 37° on a shaker bath. [^{3}H]NE (18–34 nM) was added and uptake was permitted to continue for 15 min at 37°. Each heart was then rinsed briefly in fresh Krebs buffer and incubated with 10 ml of fresh buffer (50-ml beaker) for 15 min at 37° to remove extraneuronal and loosely bound [^{3}H]NE. Subsequently, each heart was removed with a pair of forceps, blotted gently on a paper towel to remove adhering liquid, and transferred to 1.5 ml of fresh Krebs buffer in the well (approximately 2 ml capacity) of a plastic tissue culture plate. At 5-min intervals, each heart was removed and blotted as

above, and then transferred into the next well. Each plate contained four rows of six wells, sufficient for four hearts. The fifth position in each row contained 50 mM KCl, which had been substituted for an equimolar amount of NaCl. This procedure was carried out at 37° with slow shaking on a water bath. Each heart was held together by strands of connective tissue and this facilitated the transfer procedure. At the end of each experiment, a 1.0-ml aliquot was sampled from each well for the determination of released radioactivity. Additionally, the radioactivity in each heart was leeched into 3 ml of absolute ethanol by shaking at 37° for 20 min and then scintillation counting fluid was added. Fifteen ml of Bray's solution was used in some experiments and 10 ml Aquasol (New England Nuclear) in others.

In some experiments, $Al(OH)_3$ was used to isolate [^{3}H]NE from the total ^{3}H liberated from the heart. One ml of fluid from the wells of duplicate heart specimens was combined and treated with freshly precipitated $Al(OH)_3$ with a modification of the method described by Locke *et al.* [10] as follows: $Al(OH)_3$ was precipitated from 10 ml of 20% (w/v) $Al_2(SO_4)_3$ in 120 ml of distilled water, containing 480 mg ascorbic acid and 0.1 M NH_4Cl, and the suspension was adjusted to pH 8.3 with NaOH. Upon addition of 3 ml of the $Al(OH)_3$ suspension to the 2-ml experimental sample, the pH remained at 8.3, which was optimal for binding catechol compounds. The mixture was permitted to stand at room temperature for 5 min with occasional shaking, and then the precipitate was centrifuged at 700 *g* for 10 min. A 1.0-ml aliquot of the supernatant was assayed for radioactivity to determine the amount of unbound (non-catechol) compounds. The precipitate was washed once by resuspension in 4.5 ml of 95% ethanol followed by centrifugation. Finally, bound catechol compounds were eluted by adding 4.5 ml of 95% ethanol–conc. HCl (99:1) and allowing the mixture to stand at room temperature for 1 hr; samples were centrifuged at 700 *g* for 10 min to sediment the residual precipitate. Two-ml aliquots of each purified extract were assayed for radioactivity. The recovery of standard [^{3}H]NE in this procedure was 90–92 per cent.

In other experiments, the radioactivity released from the heart during stimulation with 50 mM K^+ in the presence of 10^{-3} M phenylhydrazine was subjected to thin-layer chromatographic analysis. For these experiments, eight hearts were run as usual in the presence of 10^{-3} M phenylhydrazine and then 1.25 ml of medium from the K^+-stimulated samples was combined and 20 mg ascorbic acid, 54 mg NH_4Cl and 0.5 ml of 20% (w/v) $Al_2(SO_4)_3$ were added. The pH was adjusted to 8.3 with NaOH and the purification was completed according to Locke *et al.* [10]. Thin-layer chromatographic radioassay [10] was performed with Silica gel G and sec-butanol–formic acid–water (15:3:2) as solvent.

The rate of reaction of the carbonyl reagents with pyridoxal phosphate was measured at 390 nm [11] on a Gilford model 300 spectrophotometer equipped with a flow-through cell. The reaction was run at room temperature in the same medium used to study the release of [^{3}H]NE from sliced mouse heart. The reaction was initiated by the addition of 100 μl of concentrated carbonyl reagent (prepared in distilled water) to 10 ml of the buffered pyridoxal phosphate. Sodium bisulfite at 10^{-2} M (final concentration) reacted with all of the pyridoxal phosphate to form an addition complex which exhibited no absorbancy at 390 nm; therefore, 10^{-2} M bisulfite was routinely added to aliquots of reaction mixture to distinguish the absorbance of pyridoxal phosphate from the absorbance of the reaction products with the other carbonyl reagents. The residual pyridoxal phosphate was given by the difference in readings between samples read directly and samples to which 10^{-2} M sodium bisulfite had been added.

[^{3}H]DL-norepinephrine (6.9 to 10.1 Ci/m-mole) and [^{3}H]metaraminol (6.7 Ci/m-mole) were obtained from New England Nuclear. Sodium bisulfite, hydroxylamine hydrochloride and phenylhydrazine hydrochloride were obtained from Fisher Scientific; semicarbazide hydrochloride, J. T. Baker; 2,4-dinitrophenol (2,4-DNP), Nutritional Biochemicals; carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), Sigma Chemical Co.; pyridoxal phosphate monohydrate, CalBiochem; and reserpine (Serpasil), Ciba. 6-Hydroxydopamine hydrobromide (6-OHDA) was purchased from Regis Chemical Co.; solutions were prepared in ice-cold N_2-sparged 0.9% saline. The modified Krebs–Ringer buffer consisted of 118 mM NaCl, 4.7 mM KCl, 32.0 mM sodium phosphate, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$ and, additionally, contained 5.6 mM glucose, 1.7 mM ascorbic acid and 1.3 mM EDTA.

Statistical analyses were carried out with the Student's *t*-test [12].

RESULTS

Studies with carbonyl reagents. Figure 1 shows the spontaneous efflux of tritium from sliced heart with time and the effect of stimulation with 50 mM K^+ in the presence (upper panel) and absence (lower panel) of 10^{-3} M sodium bisulfite. The spontaneous efflux diminished with time in control hearts. When sodium bisulfite was added, the spontaneous efflux initially increased with time and then exhibited a plateau between 20 and 30 min. The plateau region was used to study the effect of K^+ stimulation. The stimulus-induced efflux of tritium was increased in the presence of sodium bisulfite. After stimulation with K^+, the overflow of tritium decreased and then fell below that seen for the corresponding unstimulated samples. This effect was augmented in the bisulfite-treated samples. There was then a return toward baseline (unstimulated) values.

In addition to bisulfite, two other carbonyl reagents, namely, hydroxylamine and phenylhydrazine, increased the K^+-evoked overflow of tritium. In the experiments shown in Fig. 2, the data are the dis./min released by 50 mM K^+ (stimulated sample minus baseline value; see legend to Fig. 2). The increased overflow compared to control was 162 per cent for sodium bisulfite, 179 per cent for hydroxylamine and 249 per cent for phenylhydrazine ($P < 0.001$). In these experiments, there was an upward trend in the spontaneous efflux of tritium in the presence of phenylhydrazine or hydroxylamine. The spontaneous efflux exhibited a plateau after 20 min with hydroxylamine.

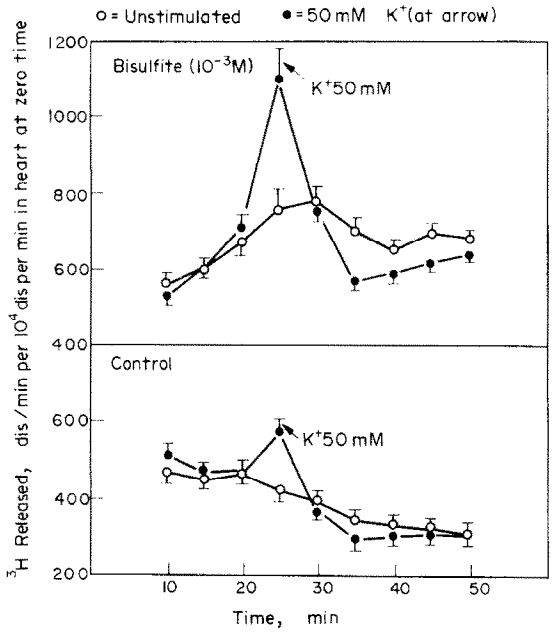

Fig. 1. Effect of 10^{-3} M sodium bisulfite on the overflow of tritium from sliced mouse heart during stimulation with 50 mM potassium ion. Individual sliced hearts were labeled with [^{3}H]NE for 15 min at 37° and then rinsed in fresh medium for 15 min. Each heart was then incubated in 1.5 ml of fresh medium and transferred at 5-min intervals into fresh medium. At the fifth interval (20–25 min), the medium contained 50 mM K^+, which was substituted for an equimolar amount of Na^+. Data are not shown for the first 5-min time interval, which was considered to be an equilibration period. Data points are the mean ± S.E.M. for six hearts. The mean heart content of [^{3}H]NE (± S.E.M.) after uptake and the initial 15-min rinse was 111,416 ± 4,433 dis./min (N = 24).

but was still increasing at 30 min with phenylhydrazine.

In other experiments, the carbonyl reagents were tested at a concentration of 2.5×10^{-4} M. There was increased stimulated overflow of tritium with hydroxylamine or phenylhydrazine, but much smaller in magnitude (viz. 56.7 and 41.4 per cent, respectively, $P < 0.02$) than that seen at 10^{-3} M. There was no significant increase in stimulated overflow of tritium with sodium bisulfite at this concentration.

One other carbonyl binding agent, semicarbazide, was also tested. This compound is known to react more slowly with aldehyde groups [13]. Semicarbazide at 10^{-3} M did not increase the K^+-stimulated overflow of tritium, nor did it alter the spontaneous efflux. The data (stimulated sample minus baseline value; see legend to Fig. 2) were as follows. The ^{3}H released by 50 mM K^+ from the control hearts was 131.9 ± 9.4 dis./min/10,000 dis./min in the heart (mean ± S.E.M.; N = 9), while that released in the presence of semicarbazide was 102.6 ± 10.6 dis./min (N = 8; −22 per cent compared to control, $P > 0.05$).

The rate of reaction of the carbonyl reagents with a representative biological aldehyde, pyridoxal phosphate (vitamin B_6), was studied spectrophotometrically (Fig. 3). The concentration of carbonyl reagent (10^{-3} M) corresponded to that used in studies with sliced mouse heart. The concentration of pyridoxal phosphate (2.5×10^{-4} M) was selected to give a convenient absorbancy (1.4 to 1.5 units) at the absorption maximum (390 nm). Phenylhydrazine reacted very rapidly with pyridoxal phosphate; the reaction was essentially complete within 1 min. Sodium bisulfite also reacted rapidly, but 23 per cent of the pyridoxal phosphate remained in equilibrium with the bisulfite addition complex. When the concentration of bisulfite was raised to 10^{-2} M, the addition complex with pyridoxal phosphate was quantitatively formed within 20 sec. Hydroxylamine reacted with pyridoxal phosphate more slowly; the reaction went to 70 per cent of completion by 2 min and to 90 per cent of completion by 5 min. Semicarbazide was the slowest reacting carbonyl reagent. Based on the length of time required for the reaction to go to 50 per cent of completion, we estimated that semicarbazide reacted only one-eighth as quickly as hydroxylamine with pyridoxal phosphate.

The radioactive material released from the hearts was subjected to purification with freshly precipitated $Al(OH)_3$ in order to bind ^{3}H-catechols and eliminate ^{3}H-*O*-methylated metabolites. Most of the tritium was bound to the $Al(OH)_3$ (Table 1). Thin-layer chromatographic analysis of pooled $Al(OH)_3$-purified extracts of samples collected during stimulation with K^+ in several experiments with phenylhydrazine confirmed that the purified tritium consisted of a single substance with an R_f corresponding to that of cold-carrier NE. During stimulation with 50 mM K^+, the percentage of $Al(OH)_3$-bound tritium rose (Table 1), indicating preferential release of [^{3}H]NE. Additionally, the percentage of bound tritium rose during the washes prior to stimulation and appeared higher

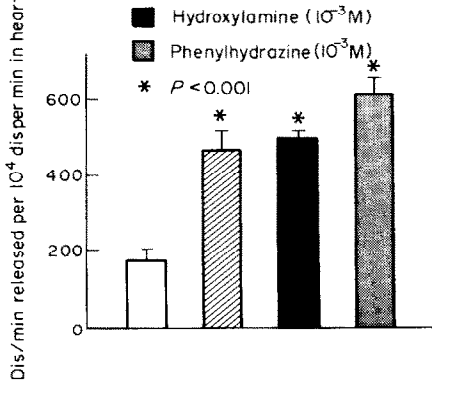

Fig. 2. Effect of carbonyl reagents (10^{-3} M) on the overflow of tritium from sliced mouse heart during stimulation with 50 mM potassium ion. Labeling with [^{3}H]NE and other details were the same as for Fig. 1. For each heart, the average of the pre- and post-stimulus samples (see Fig. 1) was subtracted from the K^+-stimulated sample to obtain the dis./min released by 50 mM K^+. Data were then expressed as dis./min released/10,000 dis./min in the heart of the pre-stimulus sample. Data are the mean ± S.E.M. for N = 7 (controls), N = 6 (bisulfite) and N = 8 (phenylhydrazine, hydroxylamine). The mean heart content of [^{3}H]NE (dis./min ± S.E.M.) after uptake and the initial 15-min rinse was 43,164 ± 2,390 (controls), 43,410 ± 4,985 (bisulfite), 44,555 ± 6,732 (hydroxylamine) and 43,132 ± 4,679 (phenylhydrazine).

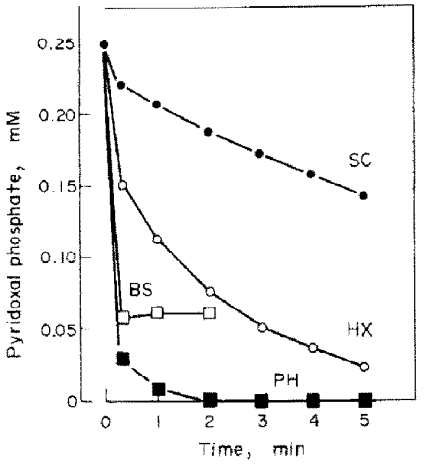

Fig. 3. Rate of reaction of the carbonyl reagents (10^{-3} M) with pyridoxal phosphate (2.5×10^{-4} M). The reaction was run at room temperature in the same medium used to study the release of [^{3}H]NE from sliced mouse heart. The absorbancy was measured at 390 nm. The amount of pyridoxal phosphate was given by the difference in readings between samples read directly and samples to which 10^{-2} M sodium bisulfite had been added to complex the residual aldehyde. Sodium bisulfite at 10^{-2} M reacted with all of the pyridoxal phosphate to form an addition complex exhibiting no absorbance at 390 nm. Residual absorbance in the samples containing the other carbonyl reagents was due to the presence of products (viz. the phenylhydrazone, semicarbazone, or oxime of pyridoxal phosphate). The phenylhydrazone had a stronger absorbance than the pyridoxal phosphate; therefore, samples were diluted 4-fold to obtain spectrophotometric readings. The initial absorbance of 2.5×10^{-4} M pyridoxal phosphate was 1.4 to 1.5 units. Abbreviations used are: semicarbazide (SC), hydroxylamine (HX), sodium bisulfite (BS) and phenylhydrazine (PH).

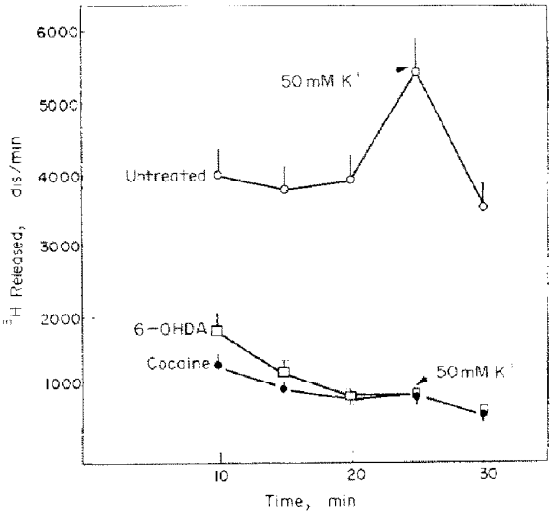

Fig. 4. Neuronal and non-neuronal release of tritium during stimulation with 50 mM K^+ in the presence of 10^{-3} M sodium bisulfite. The open circles are hearts from control (untreated) mice. The closed circles are from hearts incubated with 10^{-5} M cocaine during the uptake of the [^{3}H]norepinephrine and subsequently rinsed and incubated in medium without cocaine (see Materials and Methods). The open squares represent hearts from mice treated twice with 6-OHDA·HBr (i.v., 100 mg/kg, 40 and 20 hr). In the earlier experiments (Figs. 1 and 2), data were expressed as dis./min released/10,000 dis./min in the heart in order to account for differences in net neuronal uptake of [^{3}H]NE due to biologic variability. In this experiment (Fig. 4), changes in uptake were due to drug treatment and, therefore, data were expressed simply as released dis./min (mean ± S.E.M.). The mean heart content of [^{3}H]NE (± S.E.M.) after uptake and the initial 15-min rinse was: controls, 59,731 ± 7,536 dis./min (N = 6); cocaine-treated, 11,945 ± 662 dis./min (N = 6) and 6-OHDA, 9,646 ± 754 (N = 4).

in the prestimulus samples containing the carbonyl reagents. This may indicate that the elevated spontaneous efflux seen with sodium bisulfite in Fig. 1 represented overflow of unmetabolized [^{3}H]NE.

In order to determine if the increased overflow of [^{3}H]NE in the presence of the carbonyl reagents was derived from nerve terminals, the following experiments were run (Fig. 4). Cocaine (10^{-5} M) was added to the medium during the labeling of the heart with [^{3}H]NE in order to block uptake into the nerve terminals (see legend to Fig. 4). Subsequently, the hearts were rinsed and reincubated in cocaine-free medium. Upon stimulation with 50 mM K^+ in the presence of 10^{-3} M bisulfite, there was very little or no response. Similar results were obtained (Fig. 4) when animals were pretreated with 6-hydroxydopamine to destroy the sympathetic nerve terminals in the

Table 1. Tritium released from mouse heart labeled with [^{3}H]norepinephrine—binding to aluminum hydroxide*

Sample	Tritium recovered from aluminum hydroxide (% of total)			
	Control (N = 9)	Bisulfite (N = 3)	Hydroxylamine (N = 3)	Phenylhydrazine (N = 3)
Wash No. 1	63.8 ± 3.3	69.7 ± 4.7	66.0 ± 4.0	68.7 ± 1.2
Wash No. 2	66.9 ± 2.9	73.3 ± 3.5	69.7 ± 5.5	73.7 ± 0.6
Wash No. 3	68.6 ± 3.5	76.7 ± 3.8	73.3 ± 4.7	80.0 ± 1.0
Wash No. 4	69.1 ± 3.0	79.5 ± 3.5	76.0 ± 3.6	85.7 ± 0.6
50 mM K^+	76.0 ± 2.3	86.3 ± 2.1	83.0 ± 2.0	91.7 ± 1.5
Wash	70.3 ± 2.6	81.3 ± 3.1	77.3 ± 3.1	90.7 ± 0.6

* Data are uncorrected for recoveries which were in the range of 90–92 per cent. Data are the mean ± S.E.M. for the number of samples (N) shown in parentheses. Each sample consisted of pooled aliquots from two replicate hearts. The experimental format was as described for Fig. 1. The concentration of each reagent was 10^{-3} M.

Table 2. Tritium released from normal and reserpinized mouse hearts labeled with [^{3}H]metaraminol*

Group	Tritium released by 50 mM K^+ (dis./min/10^4 dis./min in the heart ± S.E.M.)
Normal	283 ± 21 (N = 6)
Normal + 10^{-3} M bisulfite	1102 ± 91 (N = 6)
Reserpine treated	514 ± 36 (N = 6)
Reserpine + 10^{-3} M bisulfite	1028 ± 86 (N = 8)

* Hearts from control mice and from mice pretreated with reserpine (i.p., 10 mg/kg, 16 hr) were prepared as in Fig. 1, except that [^{3}H]metaraminol (46 nM) was used in place of [^{3}H]norepinephrine. Data were analyzed as in Fig. 2. The mean heart content of [^{3}H]metaraminol after uptake and the initial 15-min rinse (dis./min ± S.E.M.) was 83,985 ± 4,033 for control hearts (N = 12) and 53,425 ± 2,718 for reserpine-treated hearts (N = 14).

heart [14, 15]. These data showed that the increase in K^+-stimulated overflow of [^{3}H]NE in the presence of the carbonyl reagents was derived from nerve terminals and not from extra-neuronal [^{3}H]NE uptake sites.

In order to determine whether or not the carbonyl reagents might be acting by blocking reuptake of a portion of the released [^{3}H]NE, the following experiment was run. Subsequent to the uptake of [^{3}H]NE into the nerves of the heart, cocaine (10^{-5} M) was added to prevent reuptake of released [^{3}H]NE. The data were analyzed as in Fig. 2. The ^{3}H released by 50 mM K^+ from the control (untreated) hearts was 148.5 ± 20.4 dis./min/10,000 dis./min in the heart (mean ± S.E.M.; N = 6), while that released after the addition of cocaine was 106.8 ± 16.3 (N = 6). Thus, cocaine failed to augment the efflux of tritium; instead, there was a somewhat diminished response to K^+ stimulation (−28 per cent, $P > 0.1$).

In other experiments, animals were depleted of endogenous NE by treatment with reserpine (10 mg/kg, 16 hr). Then, [^{3}H]metaraminol, a false transmitter which is not metabolized by monoamine oxidase, was taken up into the heart. [^{3}H]Metaraminol can enter the catecholamine-binding granules via a reserpine-resistant uptake mechanism [16, 17] and, subsequently, it can be released by field stimulation [18]. In our experiments with reserpinized preparations, sodium bisulfite (10^{-3} M) increased the K^+-stimulated release of [^{3}H]metaraminol by 100 per cent (Table 2). Results with normal hearts are presented for comparison.

Studies with oxidative phosphorylation uncouplers. The effect of the oxidative phosphorylation uncouplers, 2,4-DNP and CCCP, on K^+-stimulated release of [^{3}H]NE is shown in Fig. 5. The data are expressed as the dis./min released by 50 mM K^+ (see legend to Fig. 2). 2,4-DNP and CCCP at concentrations known to uncouple oxidative phosphorylation [19, 20] increased the stimulated overflow of tritium as compared to control by 220 and 406 per cent, respectively. The spontaneous efflux of tritium from hearts exposed to 2,4-DNP or CCCP was greater than that in control hearts at each time period prior to stimulus. However, this spontaneous overflow diminished with time in DNP-treated hearts, but increased with time in CCCP-treated hearts and reached a plateau at about 20 min.

The radioactive material released from the hearts in the presence of CCCP was purified by adsorption onto and elution from $Al(OH)_3$. Most of the tritium was bound to the $Al(OH)_3$ (Table 3). Data with control hearts from within the same experiment are presented for comparison. The results obtained with CCCP were very similar to those with the carbonyl reagents (see Table 1) in that the percentage of $Al(OH)_3$-bound tritium rose during K^+ stimulation and was higher in all drug-treated samples compared to untreated controls. The $Al(OH)_3$-purified extract of samples collected during K^+ stimulation of CCCP-treated hearts was subsequently extracted with ethyl

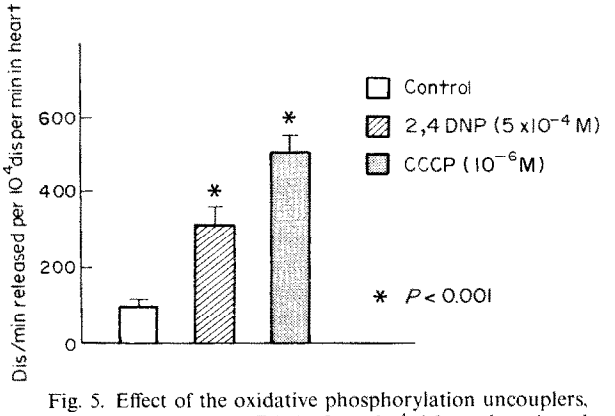

Fig. 5. Effect of the oxidative phosphorylation uncouplers, 2,4-dinitrophenol (2,4-DNP, 5×10^{-4} M) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 10^{-6} M) on the overflow of tritium from sliced mouse heart during stimulation with 50 mM potassium ion. Data were calculated as described in Fig. 2. Data are the mean ± S.E.M. for N = 12 (controls), N = 10 (2,4-DNP) and N = 10 (CCCP). The mean heart content of [^{3}H]NE (dis./min ± S.E.M.) after uptake and the initial 15-min rinse was 76,002 ± 4,002 (controls), 67,555 ± 4,807 (2,4-DNP) and 74,458 ± 4,991 (CCCP).

Table 3. Tritium released from mouse heart labeled with [^{3}H]norepinephrine—binding to aluminum hydroxide*

Sample	Tritium recovered from aluminum hydroxide (% of total) Control (N = 3)	CCCP (N = 3)
Wash No. 1	64.7 ± 0.5	73.5 ± 1.1
Wash No. 2	68.4 ± 1.4	79.9 ± 0.5
Wash No. 3	69.7 ± 1.3	82.2 ± 1.0
Wash No. 4	72.0 ± 1.6	84.2 ± 0.5
50 mM K^+	76.7 ± 1.7	88.8 ± 0.6
Wash	73.0 ± 2.7	83.4 ± 0.7

* Data are uncorrected for the recovery, which was 90 per cent. Data are the mean ± S.E.M. for the number of samples (N) shown in parentheses. Each sample consisted of pooled aliquots from two replicate hearts. The experimental format was as described for Fig. 1. The concentration of CCCP was 10^{-6} M.

acetate to estimate the amount of deaminated product [21]; only 20.2 per cent represented ^{3}H-deaminated products. Thus, the majority of the tritium released was comprised of unmetabolized NE.

As in experiments with sodium bisulfite (see Fig. 4), we also determined the effect of CCCP (10^{-6} M) on the K^+-stimulated overflow of tritium from hearts incubated with cocaine (10^{-5} M) during the uptake of [^{3}H]NE; this was done in order to evaluate possible extraneuronal release of tritium by CCCP. The results with CCCP were similar to those obtained with bisulfite, in that the response to 50 mM K^+ was markedly diminished in cocaine-treated vs untreated hearts. The data were calculated by subtracting the average of the pre- and post-stimulus samples from the K^+-stimulated sample. The amount of ^{3}H released, expressed as dis./min (mean $\pm$ S.E.M.), was 2706 ± 294 (CCCP, N = 10) and 361 ± 68.3 (cocaine + CCCP, N = 6). Therefore, the released tritium was derived from nerve terminals.

DISCUSSION

The work of several investigators has indicated the presence of feedback regulatory mechanisms that control the release of catecholamine neurotransmitters [3, 4, 6–9]. It has been proposed that these mechanisms operate through activation by released neurotransmitter of receptors that may be located either presynaptically or postsynaptically. We were interested in the possibility that 3,4-dihydroxyphenylglycolaldehyde, the product generated by monoamine oxidase acting on NE, might be involved in regulating the release of neurotransmitter from sympathetic nerves. In an attempt to trap this metabolic aldehyde, a number of carbonyl-binding agents were added to preparations of sliced mouse heart. Three agents (namely, sodium bisulfite, hydroxylamine or phenylhydrazine) increased the amount of [^{3}H]NE released during stimulation with 50 mM K^+ (Fig. 2). One other agent, semicarbazide, did not increase stimulated release. The lack of an effect by semicarbazide correlated with the known slow rate of reaction of semicarbazide with aldehydes [13], which we confirmed by studying the rate of reaction of the carbonyl reagents with pyridoxal phosphate (Fig. 3). The augmented overflow of [^{3}H]NE from hearts incubated with bisulfite was shown to be neuronal in origin. Thus, there was little or no response to K^+ stimulation in hearts from animals which had been sympathetically denervated with 6-OHDA (Fig. 4) or in normal hearts where the uptake of [^{3}H]NE into nerves had been prevented with cocaine (Fig. 4). Additionally, inhibition of reuptake of a portion of the released [^{3}H]NE was excluded as a mechanism for the increased overflow of [^{3}H]NE by experiments with cocaine (see Results).

In other experiments (to be reported separately), monoamine oxidase (MAO) inhibitors were similarly tested because these drugs would be expected to prevent formation of the aldehyde. Pargyline and a number of other MAO inhibitors increased the overflow of [^{3}H]NE by 40–80 per cent. These latter data, taken in conjunction with the current results with carbonyl reagents, were in keeping with a role for metabolic aldehydes (derived from MAO activity) in feedback regulation of neurotransmitter release. However, the experiments with [^{3}H]metaraminol (Table 2) appeared to obviate this possiblity. In these latter experiments, NE was first depleted with reserpine so that the formation of aldehyde from released endogenous neurotransmitter could be excluded. Labeling (via uptake) with [^{3}H]metaraminol then provided a nonmetabolizable amine for study. It is known that [^{3}H]metaraminol can be taken up into sympathetic nerves and storage vesicles and that it can be released as a false transmitter [18, 22]. The uptake of [^{3}H]metaraminol into catecholamine storage vesicles is not strongly affected by reserpine; that is, it enters the vesicles via the so-called "reserpine-resistant" mechanism that can be utilized additionally by dopamine [16–18], but not by NE. Farnebo [18] has shown that [^{3}H]metaraminol in reserpinized catecholamine storage vesicles of the rat iris can be released by electric field stimulation. In our experiments, there was release of [^{3}H]metaraminol from reserpinized nerves of the heart during stimulation with 50 mM K^+. When sodium bisulfite (10^{-3} M) was added, the release of tritium was augmented markedly (Table 2). This result indicated that the action of bisulfite did not require endogenous NE. Therefore, the idea concerning metabolic aldehydes appears untenable as an explanation for the actions of the carbonyl reagents.

It should be noted, however, that the release of tritium from hearts labeled with [^{3}H]metaraminol was increased by reserpinization (Table 2, absence of bisulfite). This observation was in keeping with the idea that the endogenous metabolic aldehyde (derived from NE) might be capable of exerting a negative feedback influence. Therefore, this possibility cannot be totally excluded by the current data. Alternatively, the increased overflow of tritium elicited by K^+ in reserpinized preparations may reflect other factors, such as an alteration in the properties of the amine storage vesicles caused by the binding of reserpine.

The action of bisulfite persisting in reserpinized preparations after the uptake of [^{3}H]metaraminol remains to be explained. A pertinent possibility may lie in a reaction with structural aldehydes or ketones of the membranes. If the release of transmitter requires an interaction between storage vesicles and the neuronal membrane [23], carbonyl groups in the neuronal membrane may modulate the interaction.

An alternative mode of action for bisulfite (as well as hydroxylamine and phenylhydrazine) could be a general toxic effect unrelated to a reaction with carbonyl groups. Other investigators had noted that oxidative phosphorylation uncouplers [which deprive neurons of adenosine triphosphate (ATP)] increased the overflow of transmitter during stimulation [24, 25]. Therefore, we tested two well-known oxidative phosphorylation inhibitors, 2,4-DNP and CCCP increased the spontaneous as well as the stimulated efflux of tritium as compared to control hearts (Fig. 5); the results were similar to those obtained with the carbonyl reagents (Fig. 2). 2,4-DNP produced a response that was in the range of the carbonyl-binding compounds, while CCCP, at much lower concentration, evoked the strongest response of all the compounds studied. In addition, the K^+-stimulated overflow of [^{3}H]NE in the presence of CCCP was markedly diminished in hearts exposed to cocaine to pre-

vent the accumulation of [^{3}H]NE by nerve terminals (see Results). Thus, CCCP, like bisulfite (Fig. 4), increased the release of [^{3}H]NE from neuronal sites.

Other investigators who utilized 2,4-DNP or CCCP in studies of transmitter release reported the following findings. Farnebo [24] showed a moderate increase in the overflow of [^{3}H]NE from isolated rat irises which had been incubated with 2,4-DNP and electrically stimulated; he attributed this effect to partial blockade of reuptake of NE caused by 2,4-DNP. Von Euler and Lishajko [26] reported an increase in the spontaneous release rate of NE from isolated splenic nerve granules incubated with 2,4-DNP or CCCP. They suggested that their data may have indicated that an energized state was necessary for the granules to retain bound NE. Kirpekar *et al.* [25] showed an enhanced stimulated release of NE (both electrical and K^+ stimulation) from perfused cat spleen in the presence of 2,4-DNP. These authors theorized that sympathetic nerves may depolarize during inhibition of oxidative metabolism to cause an accentuated release. In a later paper, Chang *et al.* [27] described a cardiac stimulant action in isolated guinea pig heart perfused with 2,4-DNP. They attributed their findings to a possible direct action of 2,4-DNP on extragranular stores of NE, i.e. a pool of NE which is available for immediate release and subsequently filled from the main granular stores.

It is interesting to note that most of the investigators in the studies described above did not ascribe their findings with oxidative phosphorylation uncouplers to a general toxic action of the drugs, but rather offered more specific alternative explanations. Our studies and those of the previous investigators indicate that a generalized deprivation of energy stores can increase the overflow of transmitter. This possibility also has to be considered as a possible mode of action for the carbonyl reagents. A direct study of the ATP levels in the adrenergic nerves of the mouse heart was not feasible, since it would be virtually impossible to separate the small amount of neuronal ATP from the ATP in heart muscle.

Our data indicated a good correlation between the reactivity of the carbonyl reagents and increased overflow of [^{3}H]NE. Thus, bisulfite (which makes a simple addition product) and hydroxylamine and phenylhydrazine (which react with the elimination of water) reacted rapidly with pyridoxal phosphate and also increased [^{3}H]NE overflow. On the other hand, semicarbazide, another hydrazine compound, reacted much more slowly with pyridoxal phosphate and did not effectively increase [^{3}H]NE overflow.

It may be that carbonyl reagents and oxidative phosphorylation uncouplers act at a common site. Perhaps a regulatory site on the neuronal membrane contains an essential carbonyl moiety and also has a requirement for ATP. Although the current experiments offer no clues as to the identity of such reactive carbonyl groups, they may be part of a carbohydrate residue, or they may be derived from plasmalogens, which give rise to aldehydes on mild hydrolysis. Such carbonyl groups may react reversibly with NE to form Schiff's bases. Although Schiff's bases of catecholamines can undergo ring closure to form tetrahydroisoquinoline products [28], steric factors in membranes may preclude such irreversible reactions. The reactive carbonyl groups may be part of a presynaptic regulatory mechanism that interacts with the released transmitter [9].

It is of interest to note that phenylhydrazine has been reported [29] to potentiate neuronally evoked contractions in a muscle preparation from the locust, where glutamate is the presumed neurotransmitter. Although the authors did not interpret the action of phenylhydrazine as being due to its reaction with carbonyl groups, this may be a likely explanation. If so, this may mean that the presence of regulating carbonyl moieties in neuronal membranes may be a general phenomenon.

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REFERENCES

1. R. J. Baldessarini and I. J. Kopin, *J. Pharmac. exp. Ther.* **156**, 31 (1967).
2. S. M. Kirpekar and A. R. Wakade, *J. Physiol., Lond.* **194**, 595 (1968).
3. L.-O. Farnebo and B. Hamberger, *Br. J. Pharmac. Chemother.* **43**, 97 (1971).
4. K. Starke, in *Frontiers in Catecholamine Research* (Eds E. Usdin and S. Snyder), p. 561. Pergamon Press, Great Britain (1973).
5. E. Muscholl, in *Frontiers in Catecholamine Research* (Eds. E. Usdin and S. Snyder), p. 537. Pergamon Press, Great Britain (1973).
6. J. Haggendal, in *Frontiers in Catecholamine Research* (Eds. E. Usdin and S. Snyder), p. 531. Pergamon Press, Great Britain (1973).
7. L.-O. Farnebo and T. Malmfors, *Acta physiol. scand.* (Suppl. 371), 1 (1971).
8. P. Hedqvist, in *Frontiers in Catecholamine Research* (Eds. E. Usdin and S. Snyder), p. 583. Pergamon Press, Great Britain (1973).
9. S. Z. Langer, *Biochem. Pharmac.* **23**, 1793 (1974).
10. S. Locke, G. Cohen and D. Dembiec, *J. Pharmac. exp. Ther.* **187**, 56 (1973).
11. N. Zenker, *J. med. chem.* **9**, 826 (1966).
12. G. W. Snedecor and W. G. Cochran, *Statistical Methods*, 6th Edn, p. 549 Iowa State University Press, Ames (1967).
13. R. L. Shriner and R. C. Fuson, in *The Systematic Identification of Organic Compounds*, 3rd Edn, p. 167. John Wiley, New York (1948).
14. C. Sachs, *Acta physiol. scand.* (suppl. 341), 1 (1970).
15. H. Thoenen and J. P. Tranzer, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **261**, 271 (1968).
16. P. Lundborg, *Acta physiol. scand.* **67**, 423 (1966).
17. P. Lundborg and B. Waldeck, *J. Pharm. Pharmac.* **18**, 762 (1966).
18. L.-O. Farnebo, *Biochem. Pharmac.* **20**, 2715 (1971).
19. W. F. Loomis and F. Lipmann, *J. biol. Chem.* 173, 807 (1948).
20. P. G. Heytler and W. W. Prichard, *Biochem. biophys. Res. Commun.* **7**, 272 (1962).
21. G. Cohen and S. Katz, *J. Neurochem.* **25**, 719 (1975).
22. S. J. Enna and P. A. Shore, *Biochem. Pharmac.* **21**, 1535 (1972).
23. S. Berl, S. Puszkin and W. J. Nicklas, *Science, Wash.* **179**, 441 (1973).
24. L.-O. Farnebo, *Acta physiol. scand.* (suppl. 371) 19 (1971).

25. S. M. Kirpekar, J. C. Prat and H. Yamamoto, *J. Pharmac. exp. Ther.* **172**, 342 (1970).
26. U. S. Von Euler and F. Lishajko, *Acta physiol. scand.* **77**, 298 (1969).
27. P. Chang, U. S. Von Euler and F. Lishajko, *Acta physiol. scand.* **85**, 501 (1972).
28. G. Cohen, in *Frontiers in Catecholamine Research* (Eds. E. Usdin and S. Snyder), p. 1021. Pergamon Press, Great Britain (1973).
29. R. J. Dowson and P. N. R. Usherwood, *J. Insect Physiol.* **19**, 355 (1973).