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Effect of Carbon Monoxide-Induced Hypoxia on Synaptosomal Uptake and Release of Dopamine in Rat Striatum

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We studied the effect of carbon monoxide (CO)-induced hypoxia on synaptosomal uptake and release of dopamine (DA) in rat striatum. When the rats were intoxicated at a blood level of carboxyhemoglobin (HbCO), 60-70% for 3-4hrs, [^{3}H] DA uptake was inhibited as much as 80% of control activity. This suppressed activity remained as long as 12 hrs after termination of the intoxication. After a week recovery period, the suppressed uptake activity was restored completely. When the rats were intoxicated maintaining a blood level of HbCO at 30-40% for 6-7hrs, the uptake was inhibited to 57% of the control activity and this suppressed activity was restored within 12hrs. For the rats maintaining a blood level of HbCO at 15-25% for 6-7hrs, uptake inhibition was not shown. Acute CO intoxication (at 60-70% of HbCO for 3-4 hrs) caused an increase in K^+- stimulated DA release to 147% of the control value. In conclusion, the diminished uptake and increased release of striatal DA in a CO intoxicated brain would cause an extraneuronal accumulation of DA with depletion of intraneuronal DA level, which may play a role in CO-induced hypoxic cell damage.

Key Words: Carbon monoxide-induced hypoxia, dopamine, uptake, release, striatum, synaptosomes

The primary target for carbon monoxide (CO) toxicity is considered to be the central nervous system and even very low concentrations of inhaled gas can cause a severe disruption of ongoing behavior (Laties and Merigan, 1979). The mechanism involved in delayed sequela which occurrs in a small portion of acutely intoxicated patients is not known. However it might also result from the consequences of the damaging effects of carbon monoxide to the central nervous system (Ginsburg and Romano, 1976; Jefferson, 1976). The toxicity of carbon monoxide has been attributed primarily to tissue hypoxia (Smith, 1980). The sensitivity of the nervous system to hypoxia is well known. Even short periods of hypoxia or ischemia can cause irreversible brain damage. The precise mechanism responsible for the death of hypoxic or ischemic neurons is not known. There is evidence that neurotransmitters in the synaptic regions play an important role in the pathogenesis of irreversible neuronal damage (Zervas *et al.*, 1974; Rothman, 1983; Rothman and Olney, 1986).

Uptake and release of neurotransmitters by nerve terminal are one of the major factors determining synaptic neurotransmission. Uptake of neurotransmitters by synaptosomes is an energy-dependent function that requires the fine integrity of membrane systems. Release of neurotransmitters is also a complicated integrative function. These two readily measured parameters can be used to assess neuronal transmission as well as extraneuronal concentration of neurotransmitters at the synaptic level.

The present study was conducted to investigate the effect of the CO-induced hypoxia on the active uptake and K^+- stimulated release of dopamine in the rat striatal synaptosome.

MATERIALS AND METHODS

Animals and induction of CO intoxication

Adult rats of either sex (130-150g) were divided into a control group and an acute CO-intoxicated group. The control group fasted for the same period while the acute CO-intoxicated group was under intoxication in the intoxication chamber. The intoxicated groups was divided into 3 groups.

Each group was intoxicated for 6-7 hrs maintain-

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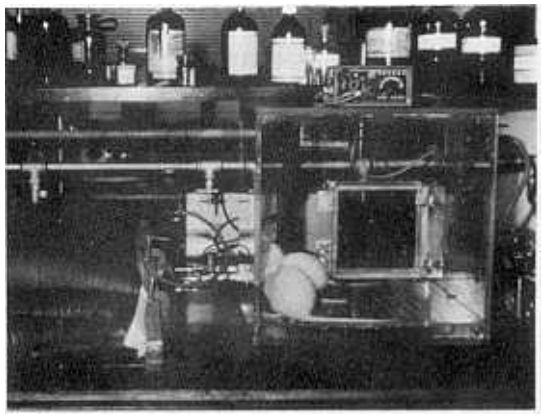
ing blood concentrations of carboxyhemoglobin (HbCO) at 15-25%, 30-40% respectively and at 60-70% of the higher concentration, intoxication was maintained only for 3-4 hrs because of the fatality. The intoxication was induced by using a gas mixture which was prepared by mixing an adequate volume of 100% CO gas and 95% O_2/5% CO_2 gas according to the differential affinity of CO and O_2 gas to hemoglobin.

The blood levels of HbCO were measured frequently by the spectrophotometric method of Tietz and Fiereck (1982) during intoxication.



Spectrophotometric determination of HbCO

Spectrophotometric determination of HbCO in the intoxicated animals was done by the method of Tietz and Fiereck (1982). The details of the method are described in our previous work (Whang *et al.*, 1985).

Preparation of neostriatal synaptosomal fraction

The rats were sacrificed and the brains were rapidly removed and washed in ice-cold saline. The striatum was dissected from the frozen brain as described by Glowinski and Iversen (1966). The striata from 1 rat weighed approx. 100mg as a wet weight. 100mg weight of wet tissue was homogenized in 0.32M sucrose to prepare a 3.3% (W/V) homogenate and centrifuged at 700g in a Sorvall RC-5B refrigerated centrifuge for 10min. The supernatant was removed and centrifuged at 27,000g for 30min.

The resulting synaptosomal pellet was suspended in 2ml of ice-cold Kreb's Ringer phosphate buffer, pH 7.4, containing 0.08mM-pargyline and 1.7mM-ascorbic acid (Heikkila *et al.*, 1975). This preparation (4°C) was used immediately for studies of synaptosomal DA uptake and release.

DA uptake assay

The DA uptake assays in both the control and intoxicated groups were carried out as described by Gershanik *et al.* (1979). 500-μl aliquots of the synaptosomes prepared previously were tranferred to each incubation flask which contained 4.5ml of ice-cold Kreb's Ringer phosphate buffer and mixed well. The buffer for the study of DA uptake contained 118mM NaCl, 15.9mM Na_2HPO_4, 4.7mM KCl, 1.8mM $CaCl_2$, 1.2mM $MgSO_4$, 0.08mM pargyline to inhibit monoamine oxidase (Heikkila, 1974). After preincubation of the samples at 37°C (0°C for the blanks) for 5min, [^{3}H] DA (39ci/mmol) was added to a final concentration of 0.2nM, 0.5nM, and 2.0nM in a total volume of 5ml. Incubation was continued for 10 min with shaking at 37°C and 0°C in each case. The incubation was stopped by decanting into ice-cold centrifuge tubes. Samples were centrifuged at 27,000g for 30min. The supernatant was discarded, the pellet was washed twice with 3ml of ice-cold saline and then 3ml of absolute ethanol was added to extract the radioactivity in the pellet. The samples were gently vortexed and after 15min at room temperature, the radioactivity in 1ml of the extract was counted in a Packard Tri-carb 300C liquid scintilation counter.

In experiments for demonstrating the effects of nomifensine, a potent inhibitor of DA uptake, and of excess amounts of unlabeled DA on the striatal [^{3}H] DA uptake, nomifensine at a concentration of 10^{-5}M was preincubated for 5 min before adding each concentration of [^{3}H] DA to the assay mixture and excess amounts (3,500×) of unlabeled DA were added to the assay mixtures simultaneously with the [^{3}H] DA. And then the samples were incubated at 37°C.

In experiments performed at 0°C, the incubation flasks were kept on ice.

The kinetic parameters were calculated according to the Lineweaver and Burk plot.

Synaptosomal [^{3}H] DA release

The DA release assay in both the control and CO-induced hypoxia groups was carried out by the combined method of Heikkila *et al.* (1985) and Hetey *et al.* (1985) 500μl aliquots of the synaptosomal fraction from both the control and intoxicated rats were preincubated with 4.5ml of Kreb's Ringer phosphate buffer as described for [^{3}H] DA uptake assay.

Then to the control synaptosomes [^{3}H] DA (39Ci/mmol) was added to a final concentration of 0.2nM and to the hypoxic synaptosomes, to a final

concentration of 1.0nM.

These concentrations were chosen since the absolute uptake values in the two groups were found to be similar at these concentrations.

The samples were then treated as previously described for the uptake assay.

These [^{3}H] DA-preloaded synaptosomes from both the control and CO-induced hypoxia groups were suspended in 1ml of either of two different release buffers: spontaneous release and K^+-stimulated release buffer. The buffer used for the spontaneous release was essentially the same as for the [^{3}H] DA uptake assay (Kreb's Ringer phosphate buffer) and for the K^+-stimulated release, Kreb's Ringer phosphate buffer containing 60mM K^+ with isomolar replacement of Na^+ and 3.6mM Ca^{++} which was a doubled concentration of the normal Kreb's Ringer phosphate buffer was used. Samples were then incubated with gentle shaking for 5 min at 37°C. Exactly 5 min after, release was terminated by transferring the tubes to ice and centrifuged at 27,000g for 30min at 4°C. The radioactivities in both the pellet and supernatant were determined as previously described.

For calculating the % dpm K^+-stimulated release of [^{3}H] DA, the % dpm spontaneous release was subtracted from the corresponding values of the K^+-stimulated fractions. Experiments were repeated 4 times for both the control and intoxicated groups.

RESULTS

Determination of the blood levels of HbCO in the CO-intoxicated rats

The blood levels of HbCO in the intoxicated rats were determined by using the standard curve of

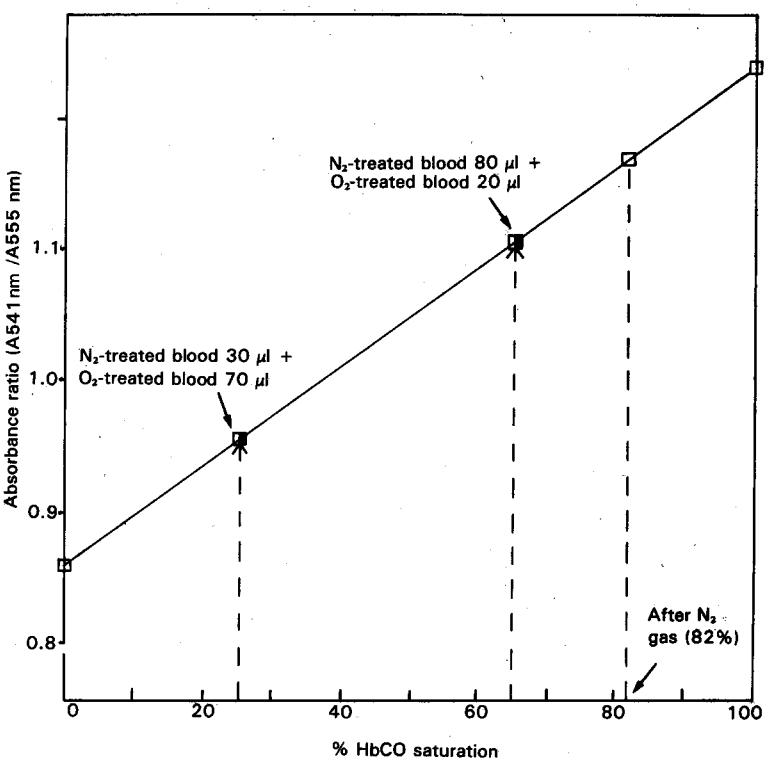


Fig. 1. Standard curve for the conversion of the 541/555 nm absorbance ratio to percent carboxyhemoglobin saturation. Fresh-heparinized blood from 5 adult rats was pooled and the preparations of the fully saturated HbO_2 and HbCO were made. The samples were immediately analyzed in triplicate by the method of Tietz and Fiereck (1982). For the intermediate points of the standard curve, 30 µl of a nitrogen treated sample was mixed with 70 µl of an oxygen-treated sample and 80µl of a nitrogen-treated sample 20 µl of an oxygen-treated sample respectivity.

HbCO obtained from the normal blood of adult rats (Fig. 1). Each group of the intoxicated rats maintained a fairly constant level of HbCO throughout the period of 6-7 hrs of intoxication (data not shown). The blood level of the HbCO in the control animals was approximately 0-5%.

Effect of CO-induced hypoxia on [^{3}H] DA uptake by rat striatal synaptosomes

[^{3}H] DA uptake was measured in striatal synaptosomes prepared from the control and CO-induced hypoxic rats. Fig. 2 demonstrates the characterization of the high affinity DA uptake system in the rat corpus striatum. The initial values of DA uptake increased proportionally with increasing the concentrations of [^{3}H] DA in the range of 0.2-2.0nM. Nomifensine (10^{-5} M), a potent inhibitor of DA uptake blocked the uptake completely and addition of unlabeled DA at a concentration of 3,500 × of labeled DA also completely blocked [^{3}H] DA uptake at 37°C.

The assay mixture incubated at 0°C also prevented uptake completely. These demonstrate the well known characterization of the DA uptake system as a temperature-sensitive and nomifensine-and excess amount of unlabeled DA-inhibited process which is specific for DA uptake (Horn, 1978; Mckillop and Bradford, 1981). Using the Lineweaver-Burk plot at multiple substrate concentrations (shown in the inset of Fig. 2), the Km and Vmax of rat striatal DA uptake carrier were found to be 7.02 × 10^{-9}M and 2.89 pmol/25mg wet weight/10min respectively (Fig. 2). The results of the effects of the various concentrations of blood HbCO in the acutely CO-intoxicated rats on the striatal [^{3}H] DA uptake are shown in Tables 1, 2 and 3 and Fig. 3.

There was a remarkable suppression of striatal DA uptake which evolved immediately after intoxication at blood levels of HbCO, 30-40% and 60-70%.

At 60-70% of HbCO (Table 1, Fig. 3), the uptake

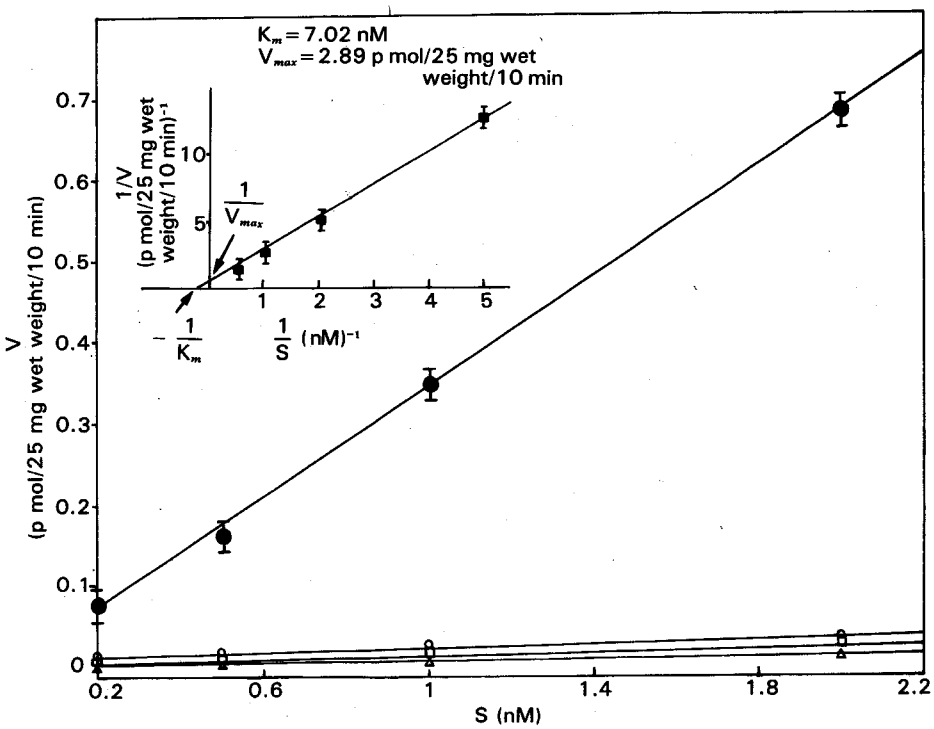


Fig. 2. Uptake of [^{3}H] DA by rat striatal synaptosomes at various concentrations of DA. The assay conditions are described in the text. ●—● : [^{3}] uptake at 37°C; ○—○ : [^{3}H] DA uptake at 0°C; □—□ : [^{3}H] DA uptake in the presence of excess amounts of unlabeled DA at 37°C; △—△ DA uptake in the presence of nomifensine at 37°C. Each point is the mean ± SEM of 3 animals. The inset shows the double-reciprocal plot of Lineweaver and Burk. Results of the kinetic analysis are shown.

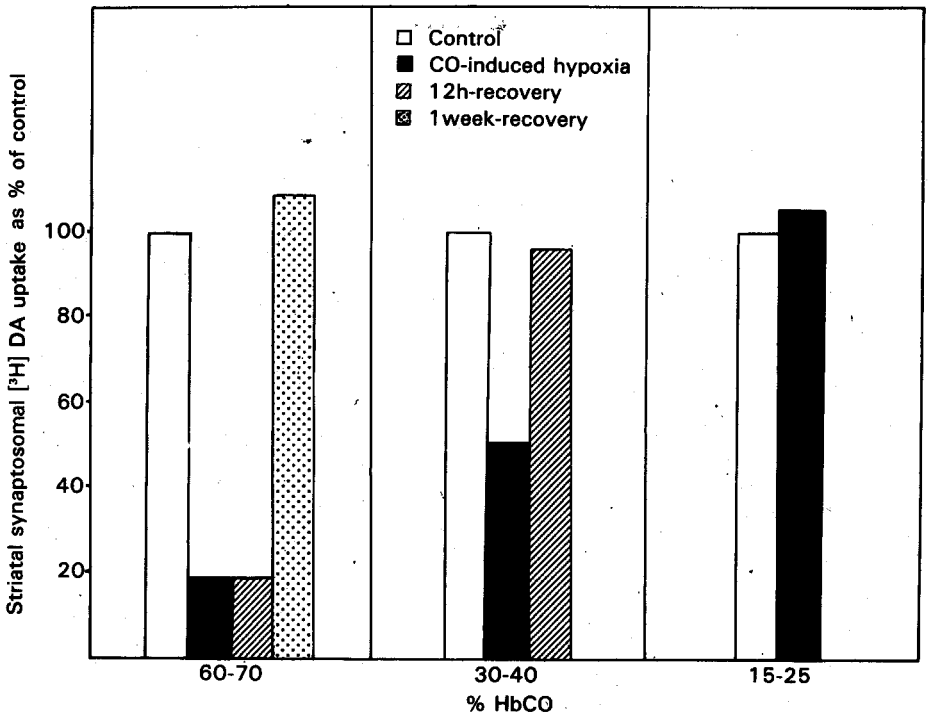


Fig. 3. *Effects of CO-induced hypoxia on [³H] DA uptake by rat striatal synaptosomes.*
Animals were intoxicated for 6-7h, maintaining blood levels of HbCO at 15-25%, 30-40% and 60-70%(3-4h), respectively. Assay conditions are described in the text. DA uptake in each group was measured immediately after intoxication. Separate experiments were carried out for the animals which recovered for 12h or 1 week from intoxication. Each bar represents the percent of control.

Table 1. Effect of CO-induced hypoxia on [³H] DA uptake by rat straital synaptosomes

	[S] nM	DA uptake (mol/25 mg wet weight/10 min)	Percent of control
Control	0.2	0.082±0.008	
	0.5	0.193±0.020	100
	2.0	0.678±0.183	
a) CO-induced	0.2	0.014±0.045	
hypoxia	0.5	0.043±0.008	22.3
	2.0	0.144±0.013	
b) 12h-recovery	0.2	0.014±0.005	
	0.5	0.040±0.006	20.7
	2.0	0.155±0.090	
c) 1 week-	0.2	0.81±0.008	
recovery	0.5	0.230±0.046	119.2
	2.0	0.849±0.116	

a. The rats were intoxicated for 3-4h, maintaining a blood level of HbCO at 60-70%.
b. The rats were allowed to recover for 12h after intoxication.
c. The rats were allowed to recover for 1 week after intoxication.
Uptake assay was done at 0.2, 0.5 and 2.0nM of substrate concentrations. The percent of control was calculated on the basis of the uptake value from 0.5nM of substrate concentration.
All values are means of 3 animals ± SD.

was inhibited as much as 80% of the control activity. This suppressed activity remained for as long as 12 hrs after 3-4 hrs intoxication regardless of appearance of the animal's arousal behavior during this period. After a week recovery period, the suppressed DA uptake activity was restored completely.

When the animals were intoxicated at a blood level of HbCO, 30-40% (Table 2, Fig. 3), for 6-7hrs, the high-affinity DA uptake was inhibited to 57% of the control activity and this suppressed activity was restored within 12hrs recovery period. When the blood level of HbCO was 15-25%, there was no inhibition of striatal DA uptake activity.

Effects of CO-induced hypoxia on the striatal synaptosomal [^{3}H] DA release

Spontaneous and K^+ evoked [^{3}H] DA releases were measured in the control and CO-intoxicated rats (maintaining a blood level of HbCO, 60-70% for 3-4hrs).

Fig. 4 shows the time course of [^{3}H] DA release by the [^{3}H]-DA-preloaded synaptosomes from the control rats. This demonstrates that the high concentrations of K^+ (60mM) and Ca^{++} (3.6mM) stimulated the total release of DA. During the first few minutes of incubation, the rate of spontaneous efflux and K^+-evoked release were similar showing that 50-60 percent of the total radioactivity in the preloaded synaptosomes were released. By 5 minutes after incubation, the K^+-stimulated release was shown to be increased by approximately 20 percent compared to the spontaneous release. Since the K^+-stimulated release of [^{3}H] DA was started at the time of 5 min of incubation, the next was started at the time of 5min

Table 2. Effect of CO-induced hypoxia on [^{3}H] DA uptake by rat striatal synaptosomes

	[S] nM	DA uptake (mol/25 mg wet weight/10 min)	Percent of control
Control	0.2	0.070±0.012	
	0.5	0.170±0.043	100
	2.0	0.656±0.209	
a) CO-induced	0.2	0.025±0.046	
hypoxia	0.5	0.073±0.008	42.9
	2.0	0.304±0.071	
b) 12h-recovery	0.2	0.074±0.015	
	0.5	0.153±0.053	90.0
	2.0	0.623±0.306	

a. The rats were intoxicated for 6-7h, maintaining a blood level of HbCO at 30-40%.
b. The rats were allowed to recover for 12h after intoxication.
Uptake assay was done at 0.2, 0.5 and 2.0nM of substrate concentration. The percent of control was calculated on the basis of the uptake value from 0.5nM of substrate concentration.
All values are means of 3 animals ± SD.

Table 3. Effect of CO-induced hypoxia on [^{3}H] DA uptake by rat striatal synaptosomes

	[S]	DA uptake (mol/25 mg wet weight/10 min)	Percent of control
Control	0.2	0.071±0.005	
	0.5	0.171±0.038	100
	2.0	0.691±0.306	
a) CO-induced	0.2	0.081±0.015	
	0.5	0.177±0.088	103.5
	2.0	0.713±0.116	

a. The rats were intoxicated for 6-7h, maintaining a blood level of HbCO at 15-25%.
Uptake assay was done at 0.2, 0.5 and 2.0nM of substrate concentration. The percent of control was calculated on the basis of the uptake value from 0.5nM of substrate concentration.
All values are means of 3 animals ± SD.

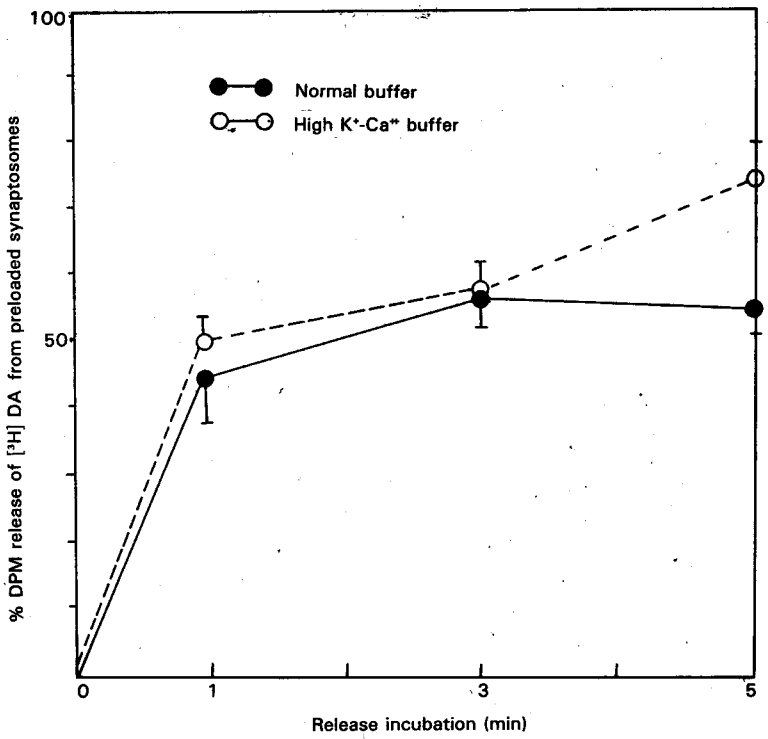


Fig. 4. *Time course and K^+-Ca^{++} dependency of [^{3}H] DA release from [^{3}H] DA-preloaded rat striatal synaptosomes. DA-preloaded synaptosomes were incubated in normal and high K^+-Ca^{++} incubation buffers at 37°C. Assay conditions are in the text. Release values at the indicated time intervals are expressed as the percent release of the total [^{3}H] DA activity in the synaptosomes. The results are means of 4 animals ± SD.*

Table 4. Effect of CO-induced hypoxia on the striatal synaptosomal release of [^{3}H] DA in the rat

	b) % dpm K^+-stimulated release of [^{3}H] DA from preloaded synaptosomes	Percent of control
Control	18 ±3.16	100
a) CO-induced hypoxia	c) 26.5±5.38	147

a. The rats were intoxicated for 3-4h, maintaining a blood level of HbCO at 60-70%.
b. The value of K^+-stimulated release was calculated by substracting the spontaneous release. The results are expressed as means of 4 animals ± SD.
c. $p<0.05$

of incubation, the next experiments (control and CO-intoxicated rats) were performed at this time point.

Table 4 shows the effect of CO-induced hypoxia on synaptosomal release of [^{3}H] DA in rat striatum. The acute CO intoxication (HbCO, 60-70% for 3-4hrs) caused on increase in K^+-stimulated DA release to 147% of the control value.

DISCUSSION

There is much evidence that the active uptake and

release system of various neurotransmitters is changed in experimental animals at certain conditions injurious to brain function such as ischemia, hypoxia-ischemia, and anoxia (Zervas *et al.*, 1974; Mrsulja *et al.*, 1976; Weinberger and Cohen, 1982; Pastuszke *et al.*, 1982; Silverstein and Johnston, 1984; Silverstein *et al.*, 1986; Freeman and Gibson, 1986).

Odarjuk *et al.* (1986) reported that DA uptake was increased and release was diminished after a single exposure to hypoxia (PO_2=11KPa) for 12 hrs. But animal models of hypoxia-ischemia that use carotid artery ligation suggested that excess DA release is an important part of the pathophysiological changes (Zervas *et al.*, 1974; Mrsulja *et al.*, 1976; Silverstein and Johnston, 1984). In vivo voltametry and in vivo brain dialysis (Clemens *et al.*, 1984; Vulto *et al.*, 1985) demonstrated large increases of extracellular DA in rats at death and during ischemia.

A nuroprotective effect of alpha-methyl-paratyrosine (AMPT, a tyrosine hydroxylase inhibitor) in experimental ischemia, which decreases brain levels of catecholamines (Weinberger *et al.*, 1985) suggests that the hypoxia-or ischemia-induced increases in extracellular DA may play a role in cell damage. Freeman and Gibson (1986) also suggested that using preparations of rat striatal slices, hypoxia and anoxia increas ed extracellular DA and this result may play a role in ischemic cell damage. One previous in vitro study by Pastuszko *et al.* (1982), suggested that the rate of [^{3}H] DA efflux from rat cerebral cortical synaptosomes was unaffected by hypoxia, although reuptake appears to be impaired. However in our experiments, we have found that in acutely CO-intoxicated rats, the active uptake of [^{3}H] DA in the striatal synaptosomal preparation was remarkably suppressed in a HbCO concentration dependent-manner (Tables 1, 2 and 3 and Fig. 3) and the K^+-stimulated release of DA was significantly increased (Table 4), and this result is consistent with Freeman and Gibson (1986). The striatal synaptosomal preparation we have used showed the well known characterizations of DA uptake system as a temperature-sensitive, and nomifensine-inhibited process (Fig. 2) (Horn, 1978; Mckillop and Bradford, 1981).

The values of Km (7.02×10^{-9}M) and Vmax (2.89 pmol/25mg wet weight/10min) for the high affinity DA uptake system in our experiment were found to be for the Km value, about 13 fold smaller and for Vmax, to be approx. 4 fold larger than the values reported by other workers (Mireylees *et al.*, 1986). Also, our system of DA release was responsive to high concentrations of K^+ and Ca^{++} (Fig. 4) as described by previous workers (Holz, 1975; Mulder and Van den Berg, 1975; Mckillop and Brandford, 1981).

Moreover, the hypoxic condition was induced by a gas mixture of carbon monoxide and oxygen gas in our experiment, which attained HbCO blood levels of 15-25%, 30-40% and 60-70% respectively. The results observed in our experiments do not reflect irreversible damage on the active reuptake and release system of DA since the function of suppressed DA uptake was recovered after some periods parallel to the depth of intoxication. However, previous findings (Zervas *et al.*, 1974; Mrsulja *et al.*, 1976; Silverstein and Johnston, 1984; Clemenset *et al.*, 1984; Vulto *et al.*, 1985; Weinberger *et al.*, 1985; Freeman and Gibson, 1986) strongly suggest that the implications of our results seem to be quite important in the development of hypoxic cell damage in the acute CO-intoxicated brain. The increased release and failure of DA uptake in the CO-intoxicated brain would result in an extraneuronal accumulation of DA with depletion of DA in the neuronal cell. The increased DA would cause long-lasting depolarization, leading to continuous cell firing and damage of the postsynaptic neurons (Wurtman and Zervas, 1974). The second implication is that the abnormally high level of DA would diffuse into the cerebrospinal fluid (CSF) and act on local blood levels just as the reported cerebral vasoconstrictor effects of norepinephrine and serotonin, causing the reduction of cerebral blood flow in experimental ischemia (Osterholm and Mathews, 1972; Toda and Fujita, 1973; Welch *et al.*, 1973). While the effects of DA on cerebral blood vessels remains to be characterized, this monoamine is known to modify with considerable potency the vascular tone in peripheral organs (Goldberg *et al.*, 1968). The possibility that brain blood flow is altered by the extraneuronal accumulation of very large amounts of DA after acute CO-intoxication merits exploration.

Another important implication of our results can be explained on the basis of other workers. It has been reported that during the reperfusion after cerebral ischemia, brain tissue could be situated on the oxidizing state, leading to the generation of activated oxygen species (Imaizumi *et al.*, 1984). Oxidation of DA can result in the generation of toxic free radicals (Graham, 1984; Imaizumi, 1984). The increased extracellular DA would undergo these types of nonenzymatic reactions possibly resulting in neuronal cell damage in the oxidizing environment such as hyperbaric oxygen therapy in the CO-intoxicated patient. A better understanding of the synaptic events of various other neurotransmitters such as, norepinephrine, serotonin and glutamate, also could provide a basis for important new specific drug

therapy for acute CO intoxication.

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