Cyanide and Central Nervous System
A Study with Focus on Brain Dopamine

by

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Cyanide and Central Nervous System

A Study with Focus on Brain Dopamine

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av

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ABSTRACT

Cyanide and Central Nervous System - a study with focus on brain dopamine.

The brain is a major target site in acute cyanide intoxication, as indicated by several symptoms and signs. Cyanide inhibits the enzyme cytochrome oxidase. This inhibition causes impaired oxygen utilization in all cells affected, severe metabolic acidosis and inhibited production of energy. In this thesis, some neurotoxic effects of cyanide, in particular, the effects on dopaminergic pathways were studied.

In a previous study, decreased levels of striatal dopamine and HVA were found after severe cyanide intoxication (5-20 mg/kg i.p.). However, increased striatal dopamine were found in rats showing convulsions after infusion of low doses of cyanide (0.9 mg/kg i.v.), at the optimal dose rate (the dose rate that gives the threshold dose).

Increased striatal dopamine synthesis was observed in rats after cyanide treatment and in vitro. Furthermore, in rat, as well as in pig striatal tissue, cyanide dose-dependently increased the oxidative deamination of 5-HT (MAO-A) and DA (MAO-A and -B) but not that of PEA (MAO-B). Thus cyanide affects both the synthesis and metabolism of dopamine.

In rats, sodium cyanide (2.0 mg/kg, i.p.) decreased the striatal dopamine D₁- and D₂-receptor binding 1 hour after injection. Increased extracellular levels of striatal dopamine and homovanillic acid were also shown after cyanide (2.0 mg/kg; i.p.). DOPAC and 5-HIAA were slightly decreased. This indicates an increased release or an extracellular leakage of dopamine due to neuronal damage caused by cyanide. Thus the effects of cyanide on dopamine D₁- and D₂-receptors could in part be due to cyanide-induced release of dopamine.

Because of reported changes in intracellular calcium in cyanide-treated animals, the effects of cyanide on inositol phospholipid breakdown was studied. Cyanide seemed not to affect the inositol phospholipid breakdown in vitro.

The effects of cyanide on the synthesis and metabolism of brain GABA were also examined. A decreased activity of both GAD and GABA-T were found in the rat brain tissue. The reduced activity of GABA-T, but not that of GAD returned to the control value after adding PLP in the incubation media. The cyanide-produced reduction of GABA levels will increase the susceptibility to convulsions, and could partly be due to GAD inhibition.

In conclusion, cyanide affects the central nervous system in a complex manner. Some effects are probably direct. The main part, however, appears to be secondary, e.g. hypoxia, seizures, changes in calcium levels or transmitter release produced by cyanide.

Keyword: CNS; cyanide; dopaminergic system; convulsions; receptor binding; tyrosine hydroxylase; monoamine oxidase; extracellular release; inositol phosphate; GABA; GAD.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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</thead>
<tbody>
<tr>
<td>3-MT</td>
<td>3-Methoxytyramine</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>5-Hydroxyindoleacetic acid</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-Hydroxytryptamine (serotonin)</td>
</tr>
<tr>
<td>6-MePtH4</td>
<td>6-Methyl-5,6,7,8-tetrahydropterine dihydrochloride</td>
</tr>
<tr>
<td>AADC</td>
<td>L-Aromatic amino acid decarboxylase</td>
</tr>
<tr>
<td>AOAA</td>
<td>Aminooxyacetic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DOPAC</td>
<td>3,4-Dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid tetra sodium salt</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
</tr>
<tr>
<td>GABA-T</td>
<td>γ-Aminobutyric acid transaminase</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>HVA</td>
<td>3-Methoxy-4-hydroxyphenylacetic acid (Homovanillic acid)</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>InsP</td>
<td>Inositol phosphate</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>3,4-Dihydroxy-L-phenylalanine</td>
</tr>
<tr>
<td>MAO</td>
<td>Monoamine oxidase</td>
</tr>
<tr>
<td>NA</td>
<td>Noradrenaline</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaCN</td>
<td>Sodium cyanide</td>
</tr>
<tr>
<td>NEN</td>
<td>New England Nuclear</td>
</tr>
<tr>
<td>NSD 1015</td>
<td>3-Hydroxybenzylhydrazine (HCL)</td>
</tr>
<tr>
<td>PC12</td>
<td>Pheochromocytoma cells</td>
</tr>
<tr>
<td>PCA</td>
<td>Perchloric acid</td>
</tr>
<tr>
<td>PEA</td>
<td>Phenylethylamine</td>
</tr>
<tr>
<td>PLP</td>
<td>Pyridoxal-5-phosphate (Vitamine B6)</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
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INTRODUCTION

Background

Cyanide is one of the most rapidly acting poisons known. Acute lethal cyanide poisoning may result from direct exposure to hydrogen cyanide or its alkali salts, and also from biotransformation of more chemically complex cyanogens. Acute cyanide intoxication will, in severe cases, cause symptoms such as respiratory arrest, fatigue, unconsciousness, convulsions, tremor and ultimately death (Egekeze and Oehme, 1980; Ballentyne, 1983; Way, 1984; D'Mello, 1987; Johnson et al., 1986, 1987). The death occurs after only a few minutes, while the signs and symptoms of cyanide poisoning appear within a few seconds after ingestion of cyanide or inhalation of cyanide vapours. Several symptoms of the acute cyanide poisoning also indicate that the brain is a major target site for cyanide (Ballantyne, 1987; Way, 1984). Acute, lethal cyanide intoxication is consistently accompanied by high concentrations of cyanide in the brain.

The main target enzyme for the action of cyanide is most probably cytochrome c oxidase, the terminal oxidase of the respiratory chain. Cyanide interacts with the ferric ion of cytochrome a3. The inhibition of cytochrome c oxidase activity will inhibit the mitochondrial electron transport system and produces a cytotoxic hypoxia in presence of normal haemoglobin oxygenation. Studies on the inhibition of cytochrome c oxidase activity in various organs following cyanide intoxication show correlation between the severity of the intoxication and the degree of inhibition of cytochrome c oxidase (Albaum et al., 1946; Isom & Way, 1976; Isom et al., 1982). Cyanide, however, also affects the activity of a number of other enzymes, e.g. decarboxylases and transaminases, (Dixon & Webb, 1958; Solomonson, 1982). The mechanisms for these effects include combination with functionally essential metal ions; formation of cyanohydrins; elimination of sulphur as thiocyanate; addition to the Schiff base aldimine with formation of aminonitrile (Hansen and Dekker, 1976).

There appear also to be regional differences in how cyanide inhibits the enzymes or in the bioavailability of the antidotes. For instance, animals given the antidotes, sodium nitrite and thiosulfate, appeared to have recovered the cytochrome c oxidase of the liver but not that of the brain (Isom & Way, 1976; Isom et al., 1982).

Neurotoxic effects of cyanide

The nervous system is considered particulary susceptible to the toxic actions of cyanide, because of its limited anaerobic metabolism, low energy reserves, and high energy demands (Brierley et al., 1976; Funata et al., 1984; Johnson et al., 1986, 1987; Yamamoto, 1990). The vulnerability of the brain to cyanide is partly due to relatively low concentrations of cyanide-metabolizing enzymes in the central nervous system (CNS) (Mimori et al., 1984). The inhibition of cytochrome c oxidase is, with high probability, the molecular basis of the central effects produced
by cyanide. But direct effects of cyanide cannot be excluded. Whatever the mechanisms, cyanide will cause impaired oxygen utilisation in all cells affected and produce severe metabolic acidosis (Yamamoto & Yamamoto, 1977). The inhibition of the brain cytochrome c oxidase by cyanide results in a neuronal cytotoxic hypoxia. In previous investigations, where the effects of cyanide were not directly studied, various conditions of hypoxia have been shown to affect a number of neuroactive substances such as neurotransmitters and cyclic nucleotides (Davis & Carlsson, 1973; Shimada et al., 1974; Gibson et al., 1978; 1981a & b; Freeman et al., 1986; Folbergrová et al., 1981). In conditions with insufficient energy production in the brain, convulsions frequently occur (Folbergrová et al., 1981; Nakanishi et al., 1991; Shukla et al., 1989). This is also the case during severe cyanide intoxication (Ballentyne, 1983; Yamamoto, 1990). It has, however, not been clarified whether the convulsions are a direct effect of cyanide or secondary to the induced cytotoxic hypoxia.

Acute administration of sodium cyanide (NaCN; 5-20 mg/kg i.p.) dramatically decreased the striatal levels of dopamine (DA) and homovanillic acid (HVA) in the rat (Persson et al., 1985). However, 3,4-dihydroxyphenylacetic acid (DOPAC) levels was not significantly changed. Thus, NaCN produced rapid and regional changes in central dopaminergic pathways. Maduh et al. (1988) found a calcium-dependent release of norepinephrine and DA from cyanide-treated PC12 cells. Furthermore, a dose-dependent release of catecholamines from these cells has also been reported (Kanthasamy et al., 1990). Cyanide also produced a marked increase in plasma catecholamines by stimulating the sympathoadrenal system (Kanthasamy et al., 1991). A transient and remarkable increase in striatal DA release was observed after application of 2 mM NaCN through a brain microdialysis membrane (Kiuchi et al., 1992).

Tursky and Sajter (1962) showed that potassium cyanide inhibited the pyridoxal-5-phosphate (PLP)-requiring enzymes glutamic acid decarboxylase (GAD) and γ-aminobutyric acid transaminase (GABA-T) in the rat brain. Increased amount of glutamic acid in cerebellum, striatum and hippocampus was seen after administration of NaCN (5-10 mg/kg; i.p.). But higher dose (20 mg/kg i.p.) decreased the levels of glutamic acid and γ-aminobutyric acid (GABA) (Persson et al., 1985). In addition, animals have been shown to have a significantly increased acetylcholine esterase activity in cerebral cortex, hippocampus and midbrain in acute cyanide intoxication (Owasoyo & Irarain, 1980).

Cyanide-induced accumulation of calcium (Ca$^{2+}$) within nervous tissue has been correlated with convulsion and tremor (Johnson et al., 1986; Yamamoto, 1990). Most likely the complex interplay between Ca$^{2+}$-accumulation and convulsions also involves the release of neurotransmitters (Nachshen & Sanches-Armass, 1987). Johnson et al. (1986) also suggest that calcium channel blocking agents may be useful in limiting the severity of centrally-mediated symptoms of acute cyanide poisoning. Furthermore, they proposed that Ca$^{2+}$ functions as a toxicogenic second messenger following cyanide-induced inhibition of adenosine triphosphate (ATP) production (Johnson et al., 1987).

Maduh et al. (1990) showed that diltiazem, which blocks calcium channels at the cell surface, prevented cyanide-induced mitochondrial swelling. Cyanide-stimulated increase in cytosolic Ca$^{2+}$ appears to activate neurotransmitter secretion as
evidenced by the granula depletion and reversal of this phenomenon by diltiazem. These observations have important implications in cyanide toxicity, since the functional correlate would be release of central transmitters producing excessive CNS firing.

Marked increase (200%) in cerebral blood flow (CBF) resulting in substantial increase in brain O₂ delivery was seen after NaCN injection (2 mg/kg; i.v.) (Lee et al., 1988 a&b). Pitt et al., (1979) and Russek et al., (1963) also found increased CBF after administration of cyanide. Permanent neurological damage by cyanide stems from inhibition of cellular metabolism and is preceded by changes in cell morphology (Brierley et al., 1976; Ashton et al., 1981; Funata et al., 1984). Changes in cell morphology are therefore important indications of neuronal lesions. A calcium channel blocker prevented cyanide-induced morphological changes in PC12 cells. These changes were probably mediated by an influx of extracellular calcium. A proposed mechanism of structural damage resulting from disruption of cell metabolism suggests that adenosine triphosphate (ATP) depletion inhibits active membrane transport of ions, producing altered ionic homeostasis and accumulation of intracellular sodium and water (Schwertschlag et al., 1986).

Furthermore, severe cyanide intoxication is known to cause symptoms and signs similar to Parkinson's disease as well as lesions in the basal ganglia (Schwab & England, 1968; Finelli, 1981; Utti et al., 1985; Carella et al., 1988; Messing & Storch, 1988; Rosenberg et al., 1989; Grandas et al., 1989). Whether such lesions can be considered specific for cyanide (directly or indirectly) is not clear.

**Dopaminergic system**

**Synthesis of dopamine**

The catecholamines are synthesized from the aromatic amino acid L-tyrosine (Figure 1). L-tyrosine is transported across the blood-brain barrier by an active transporter shared by all large neutral amino acids (Pardridge, 1977). Subsequently, tyrosine is taken up into the DA neuron by an active mechanism. The suggestion that L-tyrosine is converted in a sequence to 3,4-dihydroxy-L-phenylalanine (L-DOPA), DA, noradrenaline (NA) and adrenaline by enzymes was confirmed *in vitro* in the adrenal medulla and in adrenergic nerves by Goodall & Kirshner (1957, 1958). This work was preceded by *in vivo* studies showing adrenaline formation from L-tyrosine (Gurin & Delleuva, 1947) and also from L-DOPA and DA (Udenfriend & Wyngaarden, 1956). Tyrosine hydroxylase (TH) catalyzes the first, rate-limiting, step in the catecholamine synthesis, where L-tyrosine is hydroxylated to L-DOPA in peripheral and central catecholaminergic neurons (Nagatsu et al., 1964). Tetrahydrobiopterin is considered to be the natural cofactor for TH (Weiner, 1979).

L-DOPA is subsequently decarboxylated to DA by L-aromatic amino acid decarboxylase, AADC, (Roth et al., 1987). AADC requires pyridoxal-5-phosphate (PLP, vitamin B₆) as cofactor (Holtz & Palm, 1964). L-DOPA turnover is very rapid and the levels in brain are difficult to detect under normal condition. The
enzyme is not entirely specific, since it could also catalyze the decarboxylation of other aromatic amino acids, e.g. 5-hydroxy-L-tryptophan, L-5-HTP (Rosengren, 1960a; Lovenberg et al., 1962). Furthermore, an increase in impulse flow in DA neurons, as in other monoamine-containing neurons, results in an increase in synthesis, turnover and catabolism of DA (Roth et al., 1974).

Figure 1. The major pathways in the synthesis and metabolism of catecholamines.

Storage, release and reuptake of dopamine

DA has to be taken up by the synaptic vesicles to become available for release by nerve impulses. This process requires ATP. The vesicular uptake mechanism could be selectively inhibited, irreversibly by reserpine, or reversibly by tetrabenazine (Carlsson, 1965).

After release into the synapse, the DA is taken up by cells lining the synapase. The reuptake in the catecholaminergic neurons is the best known. This reuptake is the most important inactivation mechanisms of released catecholamines. Mazindole and nomifensine are potent inhibitors of the DA reuptake, but not selective (see Koe, 1976)
Metabolism of dopamine

The metabolism of DA occurs through enzymatic degradation. Two catabolic pathways exist, either oxidative deamination or O-methylation (Figure 1). It has been suggested that, the metabolism of DA, in the rat striatum results in approximately 80% HVA formed from DOPAC and 20% HVA from 3-methoxytyramine (3-MT) (Westerink & Korf, 1976; Westerink & Spaan, 1982). These reactions are catalyzed by monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT), respectively. Accumulation of monoamines in the brain was shown after inhibition of MAO, which indicated the importance of this enzyme for the metabolism of central monoamines (Carlsson et al., 1960). This was further supported by Rosengren, (1960b), Andén et al. (1963 a&b) and Sharman (1963). MAO catalyzes the reaction of DA with molecular oxygen and water to form the corresponding aldehyde, hydrogen peroxide and ammonia (see Blaschko, 1974). In the central dopaminergic systems, formation of acid appears to dominate, whereas in the central NA system, oxidative deamination mainly leads to formation of alcohols (Jonason, 1969). MAO is located in the outer membrane of the mitochondrion (see Greenawalt, 1972). This enzyme can be divided into two forms, MAO-A and MAO-B, which differ in substrate specificity and in sensitivity to inhibitors (Fowler & Tipton, 1984).

Regulation of the dopamine neurons

Several feedback mechanisms control the activity of DA neurons (Figure 2). The synthesis of DA is regulated via the activity of the rate-limiting enzyme TH. Short-term regulation of TH has been demonstrated to be due to several independent feedback mechanisms, including end-product inhibition (Nagatsu et al., 1964; Carlsson et al., 1976) and catecholamine receptor-mediated regulation of TH (Kehr et al., 1972; Walters & Roth, 1976). DA receptors are located both postsynaptically and presynaptically (Figure 2). The presynaptic receptors are termed "autoreceptors" and several authors have demonstrated that these receptors play a crucial role in the regulation of DA synthesis and release. Furthermore, DA synthesis and release are also dependent on the intracellular Ca$^{2+}$ levels (Roth et al., 1987).

Thus, stimulation of DA autoreceptors by DA or DA agonists e.g. apomorphine leads to a decrease in impulse flow and release of DA, while blockade of the autoreceptor increases the impulse flow and transmitter release (Andén et al., 1967; Bunney et al., 1973 a&b; Farnebo and Hamberger, 1971). In addition, the impulse flow in the nigrostriatal dopaminergic neurons is suggested to be feedback regulated also via postsynaptic DA receptors (Bunney & Aghajanian, 1976; 1978).
Figure 2. A schematic model of a dopaminergic nerve terminal. A nerve impulse results in Ca$^{2+}$-dependent release of DA. Increased impulse flow also stimulates the TH, the rate-limiting step in DA biosynthesis. Presynaptic autoreceptors modulate the synthesis and release of DA. Postsynaptic receptors in striatum regulate the activity of nigrostriatal dopamine neurons via a negative feedback.
GABAergic system

GABA is known to be an inhibitory CNS transmitter, which hyperpolarizes mammalian neurones (Curtis and Watkins, 1965). High concentrations of GABA are found in the brain and spinal cord (Ryall, 1975). The turnover of GABA, which correlates with GABAergic activity, is regulated by the GABA-producing enzymes GAD and the GABA-catabolizing enzyme GABA-T. Both enzymes utilize PLP as co-enzyme. A relationship between the inhibition of GABA synthesis and the presence of convulsions has been shown (Killam, 1957; Killam & Bain, 1957; Killam et al., 1960). Convulsants such as aminooxyacetic acid (AOAA) or derivate of PLP are believed to operate via inhibition of the GAD-activity (Tapia & Sandoval, 1971). An inhibitory effect of cyanide on the activities of GAD and GABA-T was shown by Tursky and Sajter (1962). In a preliminary study we also reported reduced GABA levels in the acute cyanide intoxication (Persson et al., 1985).

![GABAergic system diagram]

Figure 3. A schematic picture showing the reactions involved in the GABA-shunt.

GABA is synthesized from glutamate by the enzyme GAD (Figure 3). This enzyme is concentrated in the nerve terminals (Salganicoff & De Robertis, 1965; Fonnum, 1968), most probably in the cytoplasm. GAD activity is strongly related to the binding with PLP in vivo (Miller et al., 1978). In presence of low concentrations of PLP, GAD is inhibited by GABA at physiological concentrations (Porter & Martin, 1984). GABA is initially metabolized by GABA-T to succinic semialdehyde and then subsequently by succinic semialdehyde dehydrogenase to succinic acid. GABA-T binds PLP strongly.
THE PURPOSE OF THE THESIS

The purpose of this study was to increase the knowledge of the acute effects of cyanide on the CNS. In the light of our previous findings that cyanide selectively affects central dopaminergic and GABAergic systems, the intention was to reveal some of the mechanisms behind these effects.

The aim of the studies on which this thesis is based were:

- To find out, by estimating the convulsive threshold, if there is a relationship between cyanide-induced convulsions and regional brain levels of dopamine and its main central metabolites (Paper I)

- To examine the effects of NaCN on the dopamine synthesis in vivo (paper II) and on tyrosine hydroxylase in vitro. This enzyme is the rate-limiting step in the synthesis of dopamine (and other catecholamines) (Paper III)

- To study the effects of cyanide in vitro on one of the most important enzymes involved in the metabolism of monoamines, MAO (Paper IV)

- To examine the effects of cyanide on the dopamine D1- and D2-receptors (Paper V)

- To investigate in living animals the effects of cyanide on the extracellular release of dopamine and the main brain dopamine metabolites (Paper VI)

- To examine in vitro the effects of cyanide on the synthesis and metabolism of the inhibitory amino acid transmitter GABA, known to counteract convulsive activity (Paper VII)
MATERIALS AND METHODS

Animals

Male, Sprague-Dawley rats, ALAB (now Bantin and Kingman International), Sollentuna, Sweden were used throughout the studies (Paper I-VII). Their body weight were 175-300 g. The animals were housed 3 per cage. The room temperature was 21-24 °C and humidity 50±5%. Brains from pigs, Swedish Landrace, were used in two studies (Paper IV and VI).

The animal experiments have been approved by the Regional Research Ethical Committee according to National laws (SFS 1988:539, LSFS 1989:41)

Chemicals

Radiochemicals

\(^{14}\text{C}-5\text{-HT}, \left[^{14}\text{C}\right]5\text{-hydroxytryptamine binoxalate}, 56.7 \text{ mCi/mmol}^{-1}, \text{NEN, Boston, USA (Paper IV).}\)

\(^{14}\text{C}\text{-PEA}, \left[^{14}\text{C}\right]2\text{-phenylethylamine hydrochloride}, 55.5 \text{ mCi/mmol}^{-1}, \text{NEN, Boston, USA (Paper IV).}\)

\(^{14}\text{C}\text{-DA}, 3,4\left[-^{14}\text{C}\right]\text{dihydroxyphenylethylamine hydrobromide, 56.0 mCi/mmol}^{-1}, \text{Amersham, England (Paper IV).}\)

SCH 23390 [N-methyl-\(^3\text{H}\)], 87.0Ci/mmol, NEN, Boston, USA (Paper V).

Spiperone, [benzene, ring-\(^3\text{H}\)], 23.3 Ci/mmol, NEN, Boston, USA (Paper V).

Myo-[2-\(^3\text{H}\)]inositol, 10-20 Ci/mmol, Amersham, England (Paper VI).

\([1,^{14}\text{C}]\text{-GABA, 50.4 mCi/mmol, NEN, Boston, USA (Paper VII).}\]

L-[1-\(^{14}\text{C}\)]-glutamic acid, 47.3 mCi/mmol, NEN, Boston, USA (Paper VII).

Other chemicals

Cis-(z)-flupenthixol dihydrochloride, H. Lundbeck A/S (Copenhagen-Valby, Denmark) and (+)-butaclamol, Ayerst Laboratories (Toronto, Canada) (Paper V)

3-Hydroxybenzylhydrazine HCl (NSD 1015), synthesized by Dr. Bengt Magnusson, University of Umeå, Sweden (Paper II and III).

All other chemicals used were pro analysi or of higher purity.

Methods

Methods used to kill the animals

The animals were killed by exposure to high-intensity microwave irradiation, when the levels of brain transmitters were measured (Paper I, II). The exposure time was 1.5 sec., output power 4.5 kW at 2.45 GHz. This method gives a rapid inactivation of brain enzymes and a prevention of post-mortem changes in brain transmitter levels (Lenox et al., 1976). In all other studies (Paper III, IV, V, VI, VII), the animals were sacrificed by decapitation using a guillotine.
Selection and handling of brain tissue

After microwave irradiation (Paper I and II), the head was removed and cooled in a freezer -20°C for 2 min. Immediately thereafter the brain was removed from the head, and dissected on an ice-chilled Petri dish. The dissection into four different regions (striatum, frontal cortex, hippocampus and cerebellum) were performed macroscopically from both hemispheres. Because of our previous results, we used striatal tissue in Papers IV, V and VI (Persson et al., 1985). For practical reasons the whole brain was used in Papers III and VII to obtain sufficient enzyme activity.

HPLC analysis

A HPLC equipped with an electrochemical detector was used for separation and detection of L-DOPA and DA (Paper II and III), and DA, NA, HVA, DOPAC and 5-hydroxyindoleacetic acid (5-HIAA) (Paper I and VI). The utility of this system has become widely known in the last decade, since it has high sensitivity and selectivity (see Mefford, 1985). 2-methyl-3-(3,4-dihydroxyphenyl)-L-alanine and 3,4-dihydroxybenzylamine were used as internal standards in Paper (II and III) and Paper (I and VI), respectively. The columns used were packed with Nucleosil reversed phase, RP-18, (Macherey-Nagel, D-5160), 3 μm particles (Paper I, II and III). Quantitation was made by comparing surface area ratios between the endogenous compounds and the internal standard in the tissue samples and in external standards.

The same HPLC equipment but other chromatographic conditions were used in the microdialysis study (Paper VI). The reason for that was the necessity to enhance the sensitivity. The mobile phase was an aqueous solution containing 300 ml 1 M sodium dihydrogen phosphate, 7.4 ml 10 % EDTA, 0.691 g 1-octanesulphonic acid, 272 ml methanol and 1700 ml H2O, pH 4.0. Before use, the mobile phase was filtered through a Millipore filter 0.2 μm. DA, NA, DOPAC, HVA and 5-HIAA were separated on an analytical column Spherisorb S 5 ODS 1 (5 μm; 250 x 4 mm) (Söulentechnik, D-1000 Berlin 20). The quantitation was made by comparing surface area ratios between the endogenous compounds and external standards.

Convulsive threshold estimation (Paper I)

A modification of the method described by Wahlström (1966, 1978) was used. Sodium cyanide was dissolved in 0.9 % NaCl and infused at a constant rate in the tail vein until convulsions appeared. The tonic tail movement was selected as the starting point of convulsions during the whole experiment. The time for onset of convulsions was observed visually. Eleven different dose rates, but always the same volume rate (0.10 ml/min), were used. The dose needed to induce the convulsions was calculated and this dose is called the threshold dose. Dose plotted against dose rate generates a dose rate curve, where the minimum gives the optimal dose rate (Wahlström, 1966; Bolander et al., 1984).

By infusing the threshold dose at the optimal dose rate another group of animals could be divided into two groups, one with convulsions and one without
convulsions, but all infused with the same dose of NaCN. By using such a technique it is possible to separate the effects caused by the convulsions from the effects more directly caused by NaCN (Nordberg and Wahlström, 1988). The levels of DA, NA, HVA and DOPAC were measured in four different brains regions of the rats in the various groups.

In vivo estimation of dopamine synthesis (Paper II)

Tyrosine hydroxylation in vivo was measured as the short-term accumulation of L-DOPA after inhibition of the neuronal AADC (Ca.lsson et al., 1972). NSD 1015 (100 mg/kg i.p.), the AADC inhibitor, and NaCN (20 mg/kg i.p.) or saline were given 20 and 1 min, respectively, before death. DA formation after administration of its precursor L-DOPA (100 mg/kg i.p.) was also examined. NaCN (2.5 mg/kg i.p.) or saline and L-DOPA were administrated 30 min and 25 min, respectively, before death. All animals were killed by exposure to high intensity microwave irradiation.

Microdialysis (Paper VI)

Hypnorm (fentanyl citrate 0.02 mg and fluanisone 1 mg) and diazepam, given i.m. and i.p., respectively, were used as anaesthesia during the surgery. A stereotactic instrument was used and the rat head was positioned in a horizontal plane. The coordinates relative to the bregma were: A + 1.0, L - 2.2 mm and V -3.5. During the surgery, the core temperature of the rat was kept at 37.5 °C. After the surgical procedure there is an initial period of disturbed tissue function, when decreased blood flow and disturbed transmitter release can be expected (Beneviste et al., 1987; Drew et al., 1989; Osborne et al., 1990, 1991;). Therefore, the experiment started 5-10 days after the surgery. The rats were trained in the "freely moving" equipment two different times, 3 hours each, before the experiment started. During light halothane anaesthesia, the dummy probe was removed from the guide and the microdialysis probe (CMA/12; membrane length = 3 mm; outer diameter = 0.5 mm) inserted and locked into position. A mixture of 155 mM NaCl, 4 mM KCl and 1.2 mM CaCl₂ was pumped through the probe at a rate of 2 µl/min and fractions were collected every 20 minutes for a subsequent analysis of DA, NA, and amine metabolites. The period of damage release of neurotransmitters, caused by the probe insertion, is in rats 60-90 min (Kendrick, 1991). Therefore, the collection of the basal values were not begun until 100 min had passed. Sodium cyanide (2 mg/kg i.p.) was given after the ninth 20 min fraction. After the experiment, the location of the probe was controlled by examination of the striatum.

Dopamine D₁- and D₂-receptor binding (Paper V)

DA D₁-receptor: The DA D₁-receptor ligand, [³H]-SCH 23390 was incubated together with the striatal tissue (0.5 mg). Specific [³H]-SCH 23390 binding was determined as the difference between total binding and non-specific binding in parallel assays in the absence or presence of 1 µM cis(z)-flupenthixol dihydrochloride. All binding experiments were performed in triplicate.

DA D₂-receptor: The DA D₂-receptor ligand, [³H]-spiperone was incubated with striatal tissue (1 mg). Specific [³H]-spiperone binding was determined as the difference between total binding and non-specific binding in parallel assays in the
absence or presence of 1 μM (+)-butaclamol dihydrochloride. All binding experiments were performed in triplicate.

Inositol phospholipid breakdown (Paper VI)

Miniprisms (0.35 x 0.35 mm) were made from rat cerebral corticis and pig striata according to Fowler et al. (1987). Basal and potassium-stimulated phosphoinositide hydrolysis were determined by measuring the accumulation of inositol phosphates (InsP). InsP were produced by the hydrolysis of the prelabelled ([3H]-inositol) inositol phospholipids after inhibition of the inositol polyphosphatases by Li⁺. The miniprisms were incubated with sodium cyanide for 30 min before the agonists were added and further incubated for 25 min before the reaction was ended. The inositol phospholipid breakdown was measured as described by Berridge et al. (1982) and Watson & Downes (1983), with minor modifications (Fowler et al., 1987; Tiger et al., 1990).

Assay of TH activity (Paper III)

A modification of the method described by Hirata et al. (1983) was used. 40 μl of the enzyme preparation was incubated with the substrate, L-tyrosine, in a total reaction volume of 140 μl. As co-factor, 1 mM 6-methyl-5,6,7,8-tetrahydropteridine dihydrochloride (6-MePtH₄) was used, the pterin co-factor was dissolved in 2-mercaptoethanol in order to maintain the tetrahydropterin in reduced form throughout the incubation period. Iron was used as an activator metal to maximize TH activity. In our assay, catalase was added to the incubation medium in order to catalyze the breakdown of any hydrogen peroxide formed in the reaction system (Weiner, 1979). NSD 1015 was used in order to inhibit the decarboxylation of L-DOPA to DA. A cleaning procedure before injection into the HPLC was performed. The sample was passed via Amberlite CG 50 to aluminium oxide in a pH of 8.0-8.5. L-DOPA binds to aluminium oxide at this pH. L-DOPA was eluted from the aluminium oxide with 0.5 M PCA containing 0.25 % sodium bisulfite and 0.25 % EDTA. The level of L-DOPA was determined by injection 20 μl of the eluate into the HPLC system.

Assay of MAO activity (Paper IV)

MAO activity was assayed radiochemically by a conventional method (Eckert et al., 1980) with 100 μM 5-HT as substrate for MAO-A, 100 μM DA as substrate for both A and B form and 20 μM PEA as substrate for MAO-B. The diluted crude homogenate was suspended in 10 μM K-phosphate buffer, pH 7.4 (including 0.2 mg/ml ascorbate as an antioxidant) and incubated at 37 °C with the substrate in a final volume of 100 μl (4 min [PEA] or 20 min [DA and 5-HT]). The reaction was stopped by addition of 3 M HCl and cooling the tube on ice. Blank values were obtained by adding the HCl prior to incubations. Deaminated products were extracted into 6 ml toluene-ethylacetate (1/1, w/v) saturated with water and the radioactivity was counted in 10 ml of Econofluor.
Assay of GAD activity (Paper VII)

GAD activity was assayed using a method described by Wu et al. (1973). The $^{14}$CO$_2$ formed from L-(1-$^{14}$C)glutamic acid after incubation with the homogenate was absorbed in protozol. Various concentrations of NaCN were added to the incubation media before adding the enzyme. The influence of PLP on the reaction was determined, in a separate experiment, by adding an amount equimolar to the added cyanide.

Assay of GABA-T activity (Paper VII)

The radiochemical method of Hall & Kravitz (1967) was used with some modification. Radioactive GABA was incubated with the enzyme and appropriate co-factors. At the end of the incubation, succinic semialdehyde and succinate were separated from GABA by ion-exchange chromatography. Succinate and NAD were included in the incubation media to prevent metabolism of radioactive succinate. The influence of PLP on the reaction was determined, in a separate experiment, by adding an amount equimolar to the added cyanide.

Enzyme kinetics (Paper III, IV, VII)

The kinetic constants, $K_m$ and $V_{max}$ were calculated from an Eadie Hofstee plot according to Michal (1974) and $K_i$ from a Dixon plot (Paper IV, VII). In Paper III, $K_m$ and $V_{max}$ were calculated from a Lineweaver-Burk plot using linear regression analysis.

Protein measurement

The protein concentration was measured according to the method of Lowry et al. (1951; in Paper VII). A modification of the Lowry method made by Markwell et al., (1978) was used in Paper IV. The method of Bradford (1976) was used in one study (Paper III).

We have used different methods for the determination of protein concentrations, since the experiments were performed at two different laboratories, which used different methods for protein measurement.

Statistics

Two-tailed Student’s t-test was used for parametric comparisons (Papers I-II and IV-VII). Analysis of variance (ANOVA) was used to determine the differences between various treatments (Paper III, IV and VI).
RESULTS AND DISCUSSION

General remarks

In a previous study we have found a marked, dose-dependent decrease in the striatal DA levels already 1 min after administration of NaCN (5-20 mg/kg i.p.; Persson et al., 1985). In studies, where the animals were to survive for longer time periods we had to lower the dose to approximately half of lethal dose (2-2.5 mg/kg i.p.; Paper V, VI).

In our in vitro studies, concentrations from 12.5 - 800 μM NaCN were used. The levels of cyanide needed to produce neuronal death in vitro have been shown to be 10-100 times greater than those required to be lethal in vivo (Rothman, 1983; McCaslin & Yu, 1992).

The choice of striatum as the main region for investigation (Paper IV, V, VI) was based on our previous results (Persson et al., 1985). The largest effects were observed in this region. Furthermore, lesions in the basal ganglia (Finelli, 1981; Messing & Storch, 1988) similar to Parkinson's disease (Bernheimer et al., 1973) have been reported after severe cyanide intoxication in man.

Estimation of the convulsive effect of cyanide. Paper I

We have examined whether there is a relationship between cyanide-induced convulsions and changes in the levels of DA, NA and the main DA metabolites in the striatum (Persson et al., 1985). We initially determined the threshold dose and this minimal convulsive dose was found to be 0.71 mg/kg. The optimal dose rate was 1.8 mg/kg/min. By infusing at the optimal rate until the threshold dose was obtained, it was possible to differentiate the cyanide-treated rats into two groups: one with and another without convulsions.

In acute cyanide poisoning only the rats showing convulsions showed increased striatal DA levels (table 1, Paper I). Striatal NA was decreased, while the DA metabolites were increased in rats infused with cyanide at the optimal dose rate until the convulsions started. We found no significant changes of NA, DA or DA metabolites in the other regions studied.

The increase in DA levels of the striatum is apparently related to the convulsions. It has been shown that administration of L-DOPA gives a dramatic decrease in the incidence of extensor seizures (Daily & Jobe, 1984; Maynert, 1969), why convulsions hardly could be provoked by increased DA levels. The increase in DA could instead be due to a preventive or protective action against the effects of convulsions. The dose needed to induce the convulsion was only 1/4 of LD50. This non-lethal dose of cyanide probably affects the central nervous system without seriously injuring the neurons, while the decreased levels of DA seen after intoxication with high doses of sodium cyanide (5-20 mg/kg) (Persson et al., 1985) probably reflect cyanide-induced effects in seriously, maybe irreversibly damaged neurons. Furthermore, Wisler et al. (1991) suggest that an active or facilitated
transport into cells predominates at lower CN concentrations (< 10 μM), whereas passive diffusion of CN predominates at higher CN concentrations.

**In vitro and in vivo effects on tyrosine hydroxylase. Paper II-III**

Low levels of naturally occurring L-DOPA were found in all regions studied (Figure 4).

The striatal levels of L-DOPA were increased after NaCN injection at both lethal (20 mg/kg i.p.) and non-lethal (2.5 mg/kg i.p.) doses (Figure 5a). These results indicate an increased tyrosine hydroxylation after administration of NaCN.

However, the in vitro study showed an increased activity of TH after addition of 50 μM NaCN, but higher concentrations (100 and 200 μM) did not increase the TH activity. These higher concentrations were similar to those known to produce
cell death *in vitro* (Mc Caslin & Yu, 1992). In animals injected with NaCN (20 mg/kg i.p.) together with the inhibitor of neuronal AADC, NSD 1015, there was a significant increase in the rate of accumulation of striatal L-DOPA. These results indicate an increased synthesis of L-DOPA after cyanide treatment.

Lethal and non-lethal doses of NaCN had different effects on striatal DA levels. Animals injected with NaCN (20 mg/kg i.p.) showed decreased levels of striatal DA already 1 min after injection but in animals, injected with NaCN, 2.5 mg/kg i.p., 30 min before death, the DA levels were not significantly changed but tended to increase (Figure 5b).

![Figure 5b. The striatal levels of DA in rats after administration of sodium cyanide □ Controls, □ 2.5 mg/kg i.p., □ 20 mg/kg i.p. The results are expressed as nmol/g (wet weight); mean ± S.D. n=6.](image)

After inhibition of the neuronal AADC with NSD 1015, the levels of DA did not change significantly. In cyanide-treated animals decreased DA levels were observed. In the experiments, where the DA precursor L-DOPA was given, rats injected with L-DOPA (100 mg/kg i.p.) showed increased levels of DA compared with controls. Also in rats treated with NaCN (2.5 mg/kg) and L-DOPA, there was an increase in striatal DA. This increase was, however, less pronounced than in the controls. This latter finding could indicate that cyanide inhibits the neuronal AADC. However, it is not likely that NaCN increased the striatal L-DOPA by inhibition of neuronal AADC, since increased L-DOPA levels were observed also after complete inhibition of AADC by NSD 1015. The study described in Paper II indicates an increased synthesis of DA in striatum.

An increased rate of tyrosine hydroxylation in striatum as indicated by increased levels of naturally occurring L-DOPA and increased accumulation of L-DOPA after inhibition of neuronal AADC was shown in this paper. This findings could indicate an increased synthesis of DA in this region. However, the DA levels was decreased in lethal cyanide intoxication (20 mg/kg i.p.). Thus, cyanide seriously impairs the ability of neurons to utilize oxygen (Ballantyne, 1987; Jones *et al*., 1984), but appears not to inhibit TH in lethal cyanide intoxication. The results presented in Paper III also support this idea.
The effects of cyanide on MAO. Paper IV

The oxidative deamination of DA and 5-HT were increased after addition of NaCN in vitro in both rat (Figure 6a) and pig (Figure 6b) striatal tissue. Although there was a dose-dependent increase in the MAO activity, when DA was used as a substrate, the increase was smaller than that observed using 5-HT. This probably reflects that DA is oxidatively deaminated by both MAO-A and -B. Interestingly, the oxidative deamination of PEA was not significantly changed by addition of cyanide.

**Figure 6a.** The effects of different concentrations of NaCN on MAO activity in rat striatal tissue. The control values were PEA 1.16±0.12 nmol/mg prot/min; 5-HT 1.4±0.12 nmol/mg prot/min; DA 0.28±0.04 nmol/mg prot/min; (mean ± S.D.; n=9).

**Figure 6b.** The effects of different concentrations of NaCN on MAO activity in pig striatal tissue. The control values were PEA 3.23±0.15 nmol/mg prot/min; 5-HT 0.73±0.03 nmol/mg prot/min; DA 0.76±0.04 nmol/mg prot/min; (mean ± S.D.; n=6).
Thus, when the enzyme preparations were subjected to increasing concentrations of cyanide there was an increase in the ratio of MAO-A to MAO-B activity (an increase in MAO-A activity with MAO-B activity apparently being unaffected). However, the conventional technique of determining the MAO activity i.e. incubating a tissue homogenate with a relatively high concentration of the substrate (as used by us), does not give any information about the enzyme activity within the intact neurons.

However, as discussed in more detail in Paper II, the uptake of DA into the storage granula is likely to be inhibited. This means that the synthesized DA mainly will be metabolized within the neuron by MAO-A.

The effect of cyanide on dopamine D<sub>1</sub>- and D<sub>2</sub> - receptors. Paper V

A decreased number of DA D<sub>1</sub>- and D<sub>2</sub>-receptors were seen in striatum of rats treated with cyanide, 2 mg/kg i.p., 1 hour before sacrifice. Similar effects were also observed on the DA D<sub>1</sub>-receptor 15 min after treatment at the same dose. But 24 hours after administration of NaCN neither DA D<sub>1</sub>- nor DA D<sub>2</sub>-receptor binding were significantly changed compared with controls. The temporary effects on DA D<sub>1</sub>- and D<sub>2</sub>-receptor bindings observed in our study suggest a reversible action of the injected cyanide. In severe cyanide intoxication the possibility that a hypoxic state or metabolic acidosis could result in effects on the DA receptors, must be considered. However, it is not likely that inability to use available oxygen and/or metabolic acidosis had an important role in our experiments, since the dose was only 2 mg/kg (i.p.).

In Paper VI we present data showing that sodium cyanide increases the extracellular levels of DA and HVA but decreases the levels of DOPAC. The increased DA levels will probably rapidly stimulate the adjacent DA receptors. An increased DA receptor stimulation could thus explain the decrease in both DA D<sub>1</sub>- and D<sub>2</sub>-receptor binding observed in our study. Such an increased DA receptor activation should result in a decreased synthesis of DA. On the contrary, we found that administration of NaCN increased the DA synthesis (Paper II and III).

Inhibition of release or reuptake? Extracellular levels in striatum after NaCN. Paper VI

The levels of DA and HVA were significantly increased in the extracellular fluid within 20 min after injection of sodium cyanide (2 mg/kg i.p.). However, the acid metabolite, DOPAC, considered as an intraneuronal marker of the DA terminal, and the levels of the corresponding 5-HT metabolite, 5-HIAA, were significantly decreased.
Figure 7. The effect of NaCN (2.0 mg/kg i.p.) on the extracellular levels of DA, DOPAC, HVA and 5-HIAA. The basal levels were DA 0.19±0.02 pmol/fraction; DOPAC 56.50±3.91 pmol/fraction; HVA 12.10±1.61 pmol/fraction; 5-HIAA 7.42±0.46 pmol/fraction. (The basal level was presented as the mean of fraction 6-9 ± S.D.; n=6).

In the beginning of the investigation, we tried to do the experiments in anaesthetized animals, but the animals died after injection of sodium cyanide. In order to avoid the interaction with anaesthesia the rats were awake and freely moving during the experiment. The technique used in this study has been shown to cause limited tissue damage. It is also a reliable method for assessing, on time-related basis, the in vivo changes in transmitter release into synaptic cleft from intact terminals (Di Chiara, 1991). However, although data suggest that the microdialyse probe recovers an overflow from synaptic release, it has not been proved that this overflow is quantitatively related to synaptic release.

However, secretion of DA via an exocytotic process from storage vesicles is assumed to be the primary way in which DA is released from presynaptic terminals. It is known that depolarisation-induced influx of Ca^{2+} at the terminal area is critical for triggering DA release via this process (Farnebo, 1971; Philippu & Heyd, 1970). NaCN on the other hand causes disturbances in the cellular Ca^{2+} influx (Johnson, 1986; 1987). Furthermore, DA release is dependent on the Ca^{2+} concentration and will increase in a non-linear manner with increased external Ca^{2+} (Nachshen & Sanchez-Armass, 1987).

Inositol trisphosphate is known to elevate intracellular Ca^{2+} (Berridge, 1993). Furthermore, the activation of phosphatidylinositol specific phospholipase C (PLC) stimulates the breakdown of phosphatidylinositol biphosphate to diacylglycerol and inositol trisphosphate. Sodium cyanide, on the other hand, is known to cause increased Ca^{2+} levels in cells (Johnson et al., 1986, 1987; Yamamoto, 1990). But sodium cyanide did not have any measurable effects on the inositol phospholipid breakdown in vitro. Thus, this breakdown could not be responsible for the reported accumulation of calcium after cyanide treatment.

The effect of NaCN on GABA synthesis and metabolism. Paper VII

As we earlier reported GABA was decreased in cerebellum after cyanide intoxication in vivo (Persson et al., 1985). It has been suggested that a decreased GABAergic activity during cyanide intoxication could in part explain the increased
susceptibility to convulsions after cyanide poisoning (Tapia, 1975). In this study the enzymes, GAD and GABA-T, involved in the metabolism of GABA showed a decreased activity after treatment with cyanide. Similar to AADC both enzymes require PLP as co-enzyme. Furthermore, both GAD and GABA-T retain most of their PLP attached to the enzyme (Miller et al., 1978; Kim et al., 1984). However, PLP is known to interact with cyanide and form the complex PLP-CN (Bonavita, 1960). The activity of GAD was substantially inhibited also after PLP addition. GABA-T activity remained that of controls after PLP addition. This means that the decarboxylase-PLP complex is less stable than the transaminase-PLP complex, and may thus be in a dynamic equilibration of binding and dissociation, depending on experimental condition. Hence the PLP-CN complex would inhibit GAD activity by a mechanism similar to that of PLP-AOAA (Tapia & Sandoval, 1971). Furthermore, the PLP-CN complex formed in vivo could reduce the DA levels, since all decarboxylases react in a similar way and probably will be inhibited after cyanide intoxication. The reduced levels of GABA seen after cyanide intoxication could in part be due to inhibition of GAD.
Many symptoms and signs of acute severe cyanide intoxication, e.g. incoordination of movements, unconsciousness, respiratory arrest, convulsions etc., could be either direct or indirect manifestations of toxicity in the central nervous system.

Cyanide is known to cause cytotoxic hypoxia (Way, 1984; Isom & Way, 1976; Isom et al., 1982; Ballantyne, 1987). Central neurotransmitter systems have been shown to be vulnerable to hypoxia (Davis & Carlsson, 1973; Shimada et al., 1974; Gibson et al., 1978; 1981 a & b; Freeman et al., 1986). There are few reports on the acute effects of cyanide on the central neurotransmitter systems, which may help to explain the CNS symptoms described above. Hence, this thesis was designed to elucidate some of the mechanisms behind the central effects of NaCN. The cyanide-induced inhibition of energy production, will affect several energy requiring membrane processes critical to maintain a normal neurotransmission. The flow of nerve impulse may actually be reduced, cytoplasmic Ca\(^{2+}\)-levels may be increased, the reuptake process for neurotransmitters may be reduced and granular/vesicular transport of transmitters may be decreased. Depending on relative importance or sensitivity of these processes inhibition of cytochrome c oxidase by cyanide may for example cause increased transmitter release (increase in intracellular Ca\(^{2+}\) and/or reduced reuptake) and decreased transmitter release (fewer impulses or reduced granular/vesicular content). In order to induce energy failure in a limited area a very high concentration NaCN (2mM) was infused through a microdialysis probe into striatum (Kiuchi, 1992). The results suggest that suppression of ATP production by cyanide induces an abrupt and remarkable increase in dopamine release from the nerve terminals in striatum. Furthermore, in this ischemic model a massive and transient increase in the extracellular DA level was seen, followed 3 hours later by a decreased response to high K\(^+\) stimulation. Ca\(^{2+}\)-dependent mechanisms appeared, at least in part, to be involved in these phenomena (Matsumoto et al., 1993).

The increased DA synthesis seen after cyanide intoxication may be due to an increased synaptic transmission (Aitken & Braitman, 1989). Normally, DA is metabolized and the levels of DA remain rather constant, while the concentration of DOPAC and HVA increases or decreases depending on the activity of the neuron (Roth et al., 1987). Furthermore, a change in the release of DA could affect the activation of autoreceptors and/or postsynaptic receptors. The release of vesicular DA is strongly dependent on calcium (Imperato & DiChiara, 1984). The increased synthesis and release seen after non-lethal doses of cyanide could indicate a block of the presynaptic DA receptor, resulting in an increased release and extraneuronal metabolism of DA. Furthermore, spontaneous DA release is largely dependent on calcium availability but the availability of calcium is not important for the release of the DA metabolites, DOPAC and HVA (Waters et al., 1990).

By interference with Ca\(^{2+}\) (Johnson, 1986; 1987; Yamamoto, 1990) via a disturbed ATP production (Olsen & Klein, 1947; Isom et al., 1975) cyanide might affect both the granular uptake and the release of DA. Decreased levels of DA and HVA, without corresponding change in DOPAC levels, almost certainly indicate an
intraneuronal metabolism of DA that is unrelated to release. Any effect on the negative feedback system will also affect the synthesis of DA and result in changed levels of free DA within the DA neurones. The ATP-dependent granular uptake of DA could be impaired or blocked in rats treated with high doses of cyanide. The unchanged DOPAC levels in spite of increased MAO-A activity could be explained by the inhibition of the granular uptake of DA after NaCN treatment. Uptake of DA by storage granula is necessary for the neuronal release of DA (Carlsson, 1965). If this uptake is blocked or impaired, the release of DA will be decreased or maybe abolished. The synthesis of DA will continue, but the DA storage is impaired or blocked. It is possible that there is release or leakage of DA and also some reuptake of DA. Released DA is converted to HVA, probably extraneuronally through action of COMT and MAO-(B). But DA, either synthesized or reuptaken after release, in the DA neurons will be metabolized intraneuronal MAO (-A) to DOPAC (Roth et al., 1987). This could be a possible explanation to the observed unchanged striatal DOPAC levels despite of lowered DA and HVA levels in rats treated with very high doses of cyanide. Another explanation is that the metabolite, DOPAC, can diffuse relatively freely over larger distances in the brain compared to DA (Rice et al., 1985).

A major finding of this study is that low, non-lethal doses (0.8-2.5 mg/kg) had different effects on DA levels in the striatum than high, lethal doses (5-20 mg/kg). A low dose of cyanide comparable to the estimated convulsive threshold dose (Paper I), will probably affect the central nervous system without seriously injuring the neurons (Aitken & Braitman, 1989). A common denominator of these effects may accordingly not be the reduced levels of energy as discussed above. The increased levels of DA seen in striatum after infusing the threshold dose at the "optimal dose rate" were related to the convulsions. When using "low doses" not only the tissue level but also the extracellular release of DA was increased. DOPAC, the intraneuronal marker of the DA metabolism, i.e the electric activity of the DA neuron, was decreased in extracellular fluid after treatment with NaCN (2.0 mg/kg i.p.). However, striatal DOPAC levels were not significantly changed in rats treated with lethal doses of cyanide, while striatal DA and HVA were dose-dependently decreased. These findings indicate clearly that the effects of cyanide on nigrostriatal dopaminergic system are dose-dependent. At low doses (2.0 mg/kg i.p.) the increased extracellular release of DA could stimulate the adjacent DA receptors and this increased DA stimulation could thus explain the observed decrease in both DA D_1-and DA D_2-receptor binding (Paper V). Such an increased DA receptor activation should result in a decreased synthesis of DA (Kehr et al., 1972; Masserano & Weiner, 1983). We found, however, that sodium cyanide increased the DA synthesis in vivo (high or low doses) and did not inhibit the DA synthesis in vitro (Paper II and III).

Cyanide as well as stimulation of DA receptors are known to increase the CBF (Russek et al., 1963; Pitt et al., 1979; Lee et al., 1988; Edvinsson et al, 1993). Enhanced DA levels could partly be one explanation to the increased CBF observed after cyanide. However, increased DA levels were observed only in the striatum. Furthermore, a rapid increase in the levels of DA is most certainly related to an increased TH activity triggered by cessation of impulse flow in dopaminergic pathway (Walters & Roth, 1974). The decreased levels of DA seen after intoxication with high doses of sodium cyanide (5-20 mg/kg) (Persson et al., 1985)
probably reflect cyanide induced effects in seriously, maybe irreversibly damaged neurones (Maduh et al., 1990).

There are a great number of studies showing that striatum is a brain region most commonly affected by cyanide. Severe cyanide intoxication in man has been shown to produce lesions in the basal ganglia and symptoms similar to Parkinson's disease (Schwarb & England, 1968; Finelli, 1981; Utti et al., 1985; Carella et al., 1988; Messing & Stoch, 1988; Rosenberg et al., 1989; Grandas et al., 1989). There are also reports suggesting that treatment with cyanide could injure the striatum (Hicks, 1950), white matter (Hirano et al., 1967; Levine, 1967) and substantia nigra (Hicks, 1950; Funata et al., 1984). From substantia nigra the dopaminergic neurons ascend to striatum. In addition, striatum is a very dopamine-rich brain region (Bertler & Rosengren, 1959). Data also indicate that dopaminergic neurons more easily undergo degeneration than other nerve cells in brain (McGeer, 1978). Furthermore, DA itself could probably form toxic species such as DA quinone, hydrogen peroxide and superoxide (Graham et al, 1978; Cohen, 1983; Carlsson, 1986).

Parkinson's disease is one of the few clinical conditions where an increased oxidative deamination has been observed. Thus, in cerebellar cortex, as well as in pallidum, increased MAO activities have been found (Lloyd et al., 1975; Schneider et al., 1981). In addition, studies in post-mortem striatal tissues from patients with Mb Parkinson and in animals, where the nigrostriatal projection is partially destroyed, the surviving neurones accelerate their DA synthesis and release, as compared with controls with in intact nigrostriatal pathways (Rinne et al., 1971; Agid et al., 1973). This is in agreement with our findings (Paper II, IV and VI).

Both hypoxia and cerebral ischemia increase the extracellular levels of DA and decrease the extracellular levels of DOPAC and HVA (Iijima et al., 1989; Hillered et al., 1989; Masuda et al., 1990; Gordon et al., 1990; Baker et al., 1991; Kiuchi, 1992). Hypoxia as such can induce convulsions (Folbergrova et al., 1981; Nakanishi et al., 1991; Shukla et al., 1989). It does not, however, alter the levels of striatal DA, although the synthesis of striatal DA is decreased (Freeman et al., 1986). Thus hypoxia alone is unlikely to have contributed to the induction of the convolution or the other effects in dopaminergic system observed in our investigations. Whether cyanide acts directly by changing the synthesis and extracellular levels of measured amines/amine metabolites or acts indirectly by causing hypoxia, has not been completely elucidated. Hypoxia alters both the DA release from striatum and the uptake of DA into synaptosomes (Ihle et al., 1985). Furthermore, lethal concentrations of cyanide depress quickly the synaptic transmission between Schaffer collateral-commissural fibres and CA1 pyramidal cells in hippocampal slices (Aitken & Braitman, 1989). The effects of cyanide were rapidly and completely reversed upon washout. This finding supports the possibility that there is also a direct, non-metabolic effect of cyanide in the CNS.
CONCLUSIONS

In this dissertation, some studies of the neurotoxic effects of cyanide are presented. The effects on dopaminergic pathways were studied in particular. This investigation is of course not complete. There are more mechanisms to reveal by which cyanide acts. We have found, however, that cyanide interferes with the synthesis, release and metabolism of dopamine, with dopamine receptors and also with enzymes involved in the synthesis and metabolism of dopamine. Whether the effects of cyanide are direct or indirectly mediated via for example changes in brain calcium levels, hypoxia or convulsions or maybe also via cellular damage, are problems that have to be answered.

- Cyanide-induced convulsions increased the levels of DA, HVA and DOPAC in striatum of rats, treated with the optimal dose rate until convulsions appeared. The convulsive threshold dose of cyanide was related to the striatal DA levels.

- Increased synthesis of DA, measured as the naturally occurring L-DOPA as well as short-time accumulation of L-DOPA after inhibition of decarboxylase, was observed after cyanide treatment both in vivo and in vitro.

- Sodium cyanide decreased the DA D1- and D2-receptor binding in rat striatum 1 hour after the administration (2.0 m/kg, i.p.). The effects of cyanide on DA D1- and D2-receptors are probably in part due to the effect of cyanide on the release of DA.

- Cyanide produced in rat, as well as pig, striatal tissue an immediate, dose-dependent increase in the activity of MAO-A (as measured with 5-HT as substrate), but not MAO-B (as measured with PEA). A quantitatively less increase in the MAO activity was seen with DA (a substrate for both MAO-A and -B).

- Increased extracellular levels of striatal DA and homovanillic acid were found in rats within 20 and 40 min, respectively, after cyanide (2 mg/kg; i.p.) injection. This indicates an increase in the release of DA, but could also be due to a neuronal damage caused by cyanide. A slight decrease of DOPAC and 5-HIAA were also observed.

- Sodium cyanide, in doses tested, did not affect the inositol phospholipid breakdown in vitro. The breakdown of inositol phospholipids appears not to be responsible for the accumulation of cytosolic calcium.

- Sodium cyanide decreased the activity of GAD and GABA-T in vitro. The reduction of brain GABA levels observed in the in vivo experiments could thus partly be due to GAD inhibition.
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