GABA SHUNT ENZYMES AND THE RELATIONSHIP WITH MORPHINE ABSTINENCE

PROEFSCHRIFT

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Oan Anne Dirk en Janneke Minke.

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CONTENTS

1. INTRODUCTORY SECTION

2. BIOCHEMICAL SECTION

3. BEHAVIOURAL SECTION

4. GENERAL DISCUSSION

ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
AOAA	aminooxyacetic acid
BIC	bicuculline
CNS	central nervous system
DABA	2,4-di-aminobutyric acid
DPA	di-n-propylacetate
dpm	desintegrations per min
DRP	dorsal root potential
DRR	dorsal root reflex
GABA	γ-aminobutyric acid
GABA-transaminase	4-aminobutyrate:2-oxoglutarate aminotransferase E.C. 2.6.1.19)
GAD	glutamate decarboxylase
glutamate decarboxylase	1-glutamate-1-carboxy-lyase (E.C. 4.1.1.15)
HA-966	1-hydroxy-3-amino-pyrrolidone-2
LHA	lateral hypothalamic area
LPH	lipotropic hormone
MOR	morphine
3-MPA	3-mercaptopropionate
MSH	melanocyte stimulating hormone
NAL	naloxone
NH-DABA	4-N-hydroxy-2,4-diaminobutyrate
$O.D._{340}$	optical density at 340 nm
PAD	primary afferent depolarization
p-HBA	para-hydroxybenzaldehyde
PIC	picrotoxine
PLP	pyridoxal 5′-phosphate
PLP-kinase	ATP:pyridoxal 5′-phosphotransferase (E.C. 2.7.1.35)
SAL	saline
SSA	succinic-semialdehyde
SSA-dehydrogenase	succinic semialdehyde-NAD-oxidoreductase (E.C. 2.1.16)
TSC	thiosemicarbazide

1. INTRODUCTORY SECTION

1.1. GENERAL INTRODUCTION

Selective inhibition of the rate-limiting step in the degradation of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) might be of potential use in the treatment of many neurological or psychiatric disorders since it might correct a central GABA deficiency. Alternatively, as such diseases have not been correlated convincingly with changes in the GABA-ergic system, inhibition of this rate-limiting step by specific drugs in clinical trials could be useful in demonstrating the existence of a GABA deficiency in these disturbances. The study of effects of drugs exerting a therapeutic action in specific neurological or psychiatric disorders on the rate-limiting enzyme in GABA degradation might also be useful.

Therefore, the primary object of this thesis has been to find the rate-limiting step in GABA degradation. GABA is degraded by the consecutive action of two enzymes: GABA-transaminase and SSA-dehydrogenase. It is almost generally believed that GABA-transaminase is rate-limiting in GABA degradation. As a consequence, SSA-dehydrogenase has been almost completely ignored as a likely candidate for a regulatory function in the GABA shunt. Based on *in vitro* experiments it is suggested in this thesis that SSA-dehydrogenase may have a regulatory function in the GABA shunt.

To test this hypothesis *in vivo* it became of crucial importance to find a behavioural correlate of increased GABA-ergic activity in the rat. Such a behavioural correlate might be the increased locomotor activity and quasi-morphine abstinence behaviour observed after administration of di-n-propylacetate (DPA) to the rat. This drug is used in the treatment of petit mal epilepsy (SIMON & PENRY, 1975) and probably acts via inhibition of SSA-dehydrogenase (HARVEY et al., 1975; ANLEZARK et al., 1976). The special character of the behaviour observed after administration of DPA suggests that an increased GABA-ergic activity might be related to the well known morphine abstinence syndrome. Therefore, some studies have been conducted with morphine to demonstrate this relationship. In addition, this DPA-induced abstinence behaviour has been studied pharmacologically to demonstrate its relationship with an overactive GABA-ergic system *in vivo*.

This thesis is subdivided into four sections. The introductory section contains a review of the biochemical and pharmacological aspects of the GABA-ergic system (chapters 1.2 and 1.3). It is, by purpose, not a complete review of the available literature. For such information the reader is referred to three books where the available information about GABA function in the CNS of vertebrates and

invertebrates is thoroughly discussed by experts in the fields of neurochemistry, pharmacology and electrophysiology (IVERSEN et al., 1975; BERL et al., 1975; ROBERTS et al., 1976). Some aspects which bear special importance for the experiments described in this thesis are discussed in chapter 1.4, where the current knowledge of the regulation of GABA degradation is summarized. The acute and chronic effects of opiates, the opiate receptor, the opioid peptides and their possible relationship with the GABA-ergic system are discussed in chapters 1.5 to 1.11.

The second section contains the results of biochemical experiments with the enzymes of the GABA shunt obtained from rat brain homogenates or the bacterium *pseudomonas fluorescense*. A biochemical concept for the regulation of GABA degradation via changes in affinity of the different substrates for the two enzymes involved in GABA degradation is proposed. This section also contains experiments describing the effect of monovalent and bivalent cations on GABA-transaminase and SSA-dehydrogenase activity. In addition, experiments showing the differential effects of some GABA analogues on the three GABA shunt enzymes are discussed.

The third section is devoted to the behavioural aspects of the GABA-ergic system. The use of the DPA-induced abstinence behaviour as a correlate of an increased GABA-ergic activity is described. Furthermore, the relationship of this behaviour to the acute and chronic effects of opiates is discussed, while a model for the effect of opiates on GABA-ergic neurons is presented.

The last section contains a general discussion where the findings of the biochemical and behavioural section are discussed. The individual chapters also contain a discussion section where the findings of that particular chapter are evaluated.

1.2. INTRODUCTION TO THE BIOCHEMISTRY AND PHARMACOLOGY OF GABA

GABA is an inhibitory neurotransmitter with a simple structure but with a complicated mode of action. Since it is involved in intermediary metabolism as well as neurotransmission, two separate lines of GABA research have originated. For example, the neurochemist Heinrich Waelsch once discouragingly remarked that GABA was probably a metabolic wastebasket, while Eugene Roberts was the first to wonder whether the presence in the grey matter of the central nervous system of uniquely high concentrations of GABA and the enzyme which forms it from glutamic acid has a direct or indirect relationship to transmission of the nerve impulse in this tissue (ROBERTS, 1956 and 1976). The involvement of GABA in intermediary metabolism (the net reaction of the GABA shunt is oxidative decarboxylation of 2-oxoglutaric acid into succinic acid, Fig. 1.2.1) is an important but complicating factor distinguishing GABA from other recognised neurotransmitters.

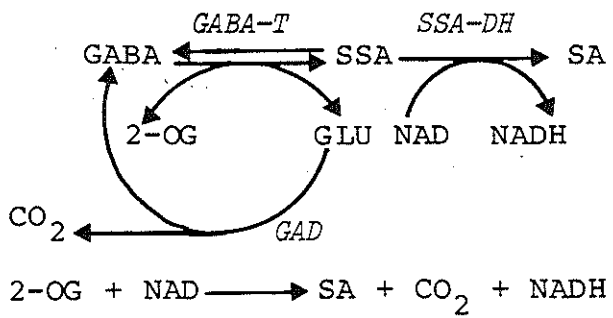


Fig. 1.2.1. The net reaction of the GABA-shunt is oxidative decarboxylation of 2-oxoglutaric acid.

1.2.1. The major pathway

GABA is synthetised from glutamate by decarboxylation. The enzyme responsible for this reaction is glutamate decarboxylase (1-glutamate 1-carboxy-lyase, E.C. 4.1.1.15). Two different forms of glutamate decarboxylase have been described which have different properties and different localizations in subcellular fractions as well as tissues. The enzyme obtained from nerve endings containing fractions is inhibited by amino-oxyacetic acid, while a second enzyme often referred to as GAD II is activated by carbonyl trapping agents and has otherwise different properties than the so called GAD I. The enzyme GAD II

appears to be similar in many respects to the glutamate decarboxylase obtained from non-neuronal tissue. The existence of GAD II has been questioned recently by many authors (MILLER & MARTIN, 1973; DRUMMOND & PHILLIPS, 1974; GONNARD & WICKER, 1974; MARTIN & MILLER, 1976) and it has been suggested that the existence of this enzyme is based on the liberation of $^{14}CO_2$ from impurities present in commercially available radioactive glutamate. The presence of glutamate decarboxylase activity in non-neuronal tissue such as heart and kidney has been reinvestigated using purified substrate and by measuring both CO_2 and GABA. These products are formed stoichiometrically suggesting that at least the enzyme obtained from non-neuronal tissue is definitely present (WU, 1976). Glutamate decarboxylase requires the presence of pyridoxal 5'-phosphate (PLP) as a cofactor and its activity is closely correlated with the availability of this cofactor (TAPIA et al., 1969). PLP is synthetised from pyridoxal and ATP by the enzyme pyridoxal phosphokinase (ATP: pyridoxal 5'-phosphotransferase, E.C. 2.7.1.35).

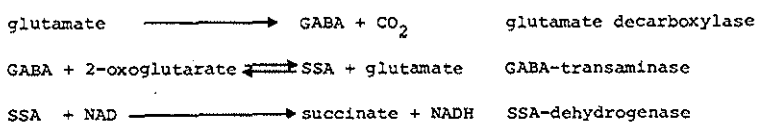


Fig. 1.2.2. The enzymatical conversions of the GABA-shunt.

GABA is degraded by the consecutive actions of two enzymes: GABA-transaminase (4-aminobutyrate: 2-oxoglutarate aminotransferase, E.C. 2.6.1.19) and SSA-dehydrogenase (succinic semialdehyde-NAD-oxidoreductase, E.C. 1.2.1.16). GABA-transaminase converts GABA into succinic semialdehyde (SSA) concurrently with the conversion of 2-oxoglutarate into glutamate, while SSA-dehydrogenase oxidizes the SSA formed into succinate by a NAD-dependent dehydrogenation reaction. The net reaction of the GABA shunt is oxidative decarboxylation of 2-oxoglutarate into succinate and the GABA shunt can therefore be considered as a bypass of the citric acid cycle (Fig. 1.2.1). The transamination of GABA is dependent on the presence of PLP, which is tightly bound on the apoenzyme. As a consequence, GABA-transaminase is completely saturated with PLP and not very sensitive to changes in the amount of available PLP. On the other hand, PLP is only weakly bound to glutamate decarboxylase and the enzyme is not completely saturated with PLP in many brain regions. Therefore, GABA synthesis is very sensitive to changes in the level of PLP. A summary of the enzymatical steps involved in GABA synthesis and degradation is given in Fig. 1.2.2.

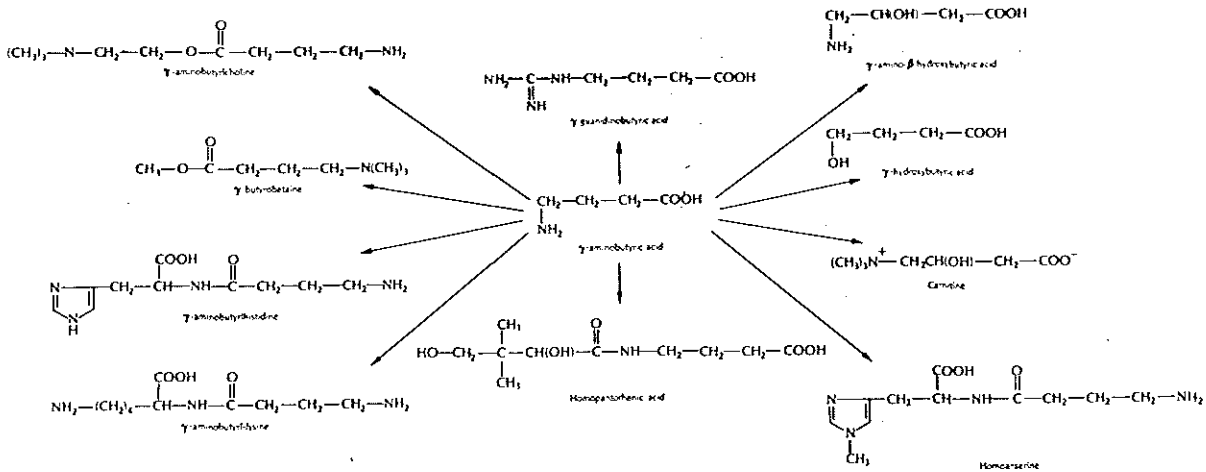


Fig. 1.2.3. Minor degradative pathways for GABA (from COOPER et al., 1974).

1.2.2. Minor pathways

The products of GABA metabolism are summarized in Fig. 1.2.3 and include 4-aminobutyricholine, 4-butyrobetaine, 4-aminobutyrylhistidine, 4-aminobutyryllysine, etc. No studies have been made to elucidate the relation of these minor products of GABA metabolism with functional aspects of the GABA-ergic system (COOPER et al., 1974). The main products of GABA metabolism (succinate and SSA) do not leave the brain, but are further oxidized in the citric acid cycle.

1.2.3. Succinic semialdehyde

SSA is probably the only degradation product of GABA that may have some relationship with the functional state of the GABA-ergic system, because SSA is in rapid equilibrium with GABA via the transamination reaction catalyzed by GABA-transaminase. Measurements of SSA concentration in the brain during different metabolic states of the GABA system have not been reported. This possibility seems to be entirely neglected, for reasons that will be discussed later. When studying the products of degradation of serotonine, dopamine or noradrenaline, a totally different situation exists, since the metabolites formed by oxidation and methylation can be easily detected in brain, cerebrospinal fluid or urine. These metabolites have therefore been used successfully to characterize the functional state of these neurotransmitter systems. A situation more or less comparable with that of GABA exists with acetylcholine. Acetate and choline, the products of acetylcholine degradation, can also be formed via alternative pathways and therefore do not reflect the functional state of the cholinergic system.

1.2.4. GABA concentration

Measurement of the concentration of GABA in the brain is possible and has been used extensively to study the functional state of the GABA-ergic system. From studies with other neurotransmitter systems it is well known that the concentration of a neurotransmitter is one of the worst correlates of changes in the functional state of such a neurotransmitter system. Wood and co-workers have repeatedly described a formula predicting the state of excitability of the brain in relation to evoked changes in GABA concentration, GABA synthesis (glutamate decarboxylase activity) and GABA degradation (GABA-transaminase activity) (WOOD, 1975). Using this formula the occurrence of convulsions could be predicted, indicating that these three factors should be considered as a whole in

order to interpret changes in the functional state of the GABA-ergic system correctly. Moreover, these authors and others have clearly demonstrated that, independently of the concentration of GABA, convulsions could be evoked by inhibiting glutamate decarboxylase activity by 35% or more (WOOD, 1975; TAPIA et al., 1975). This finding indicates that changes in GABA concentration are not followed by parallel changes in GABA available at its receptor sites. Therefore only measuring the concentration of GABA in the brain is of little value for the interpretation of the dynamics of the GABA-ergic system and its relation to functional activity.

1.2.5. The pharmacology of GABA

Recently, CHASE & WALTERS (1976) have made an excellent review of the pharmacological approach to the manipulation of the GABA-ergic system. They have considered precursor administration, biosynthesis modification, altered metabolism, uptake studies and receptor interactions as a possible way of interfering with the function of GABA *in vivo* (Fig. 1.2.4). Without repeating this review extensively, some remarks are relevant for this introduction. Because the

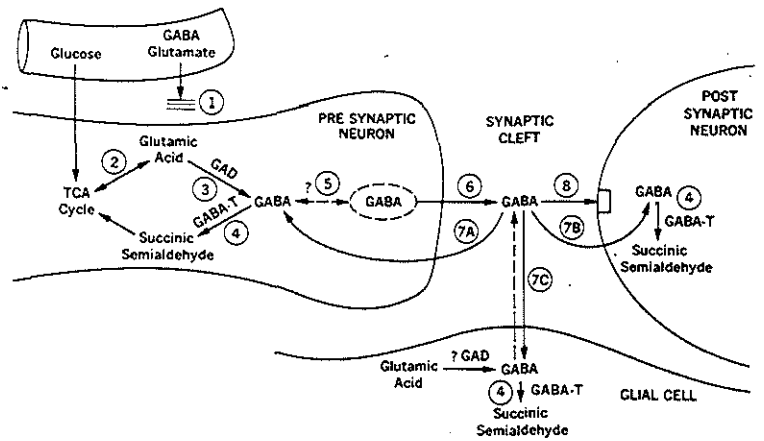


Fig. 1.2.4. Potential sites at which drugs may act to influence GABA-mediated synaptic function in the memmalian CNS. 1. blood-brain barrier transfer; 2. precursor availability; 3. biosynthesis; 4. catabolism; 5. intraneuronal storage; 6. release; 7. uptake into: A. presynaptic terminals; B. postsynaptic neurons; C. perisynaptic glia; 8. receptor interactions (from CHASE & WALTERS, 1976).

activity of glutamate decarboxylase is probably rate-limiting for GABA synthesis the administration of the GABA precursor, glutamate, does not affect the GABA system appreciably. In addition, the low liposolvibility of GABA, the presence of an enzymatical blood-brain barrier consisting of GABA-transaminase and SSA-dehydrogenase, together with the existence of an efficient GABA uptake in glial and neuronal elements, combine to completely prevent GABA penetration into the brain.

1.2.6. GABA synthesis

The enzyme catalyzing the rate-limiting step in GABA synthesis, glutamate decarboxylase, is an important site of action for drugs that influence GABA levels. Again the situation for GABA is complicated. It is possible to inhibit the synthesis of noradrenaline, dopamine, serotonine or acetylcholine by specific drugs, and to study the dynamics of these neurotransmitters by measuring the rate of depletion. Inhibition of GABA synthesis can be used as a tool to study the relation between GABA synthesis and brain excitability. However, this method has very important limitations because the behavioural correlate of this inhibition, viz. convulsions, itself affects the dynamics of the GABA-ergic system.

1.2.7. Pyridoxal 5′-phosphate

Many drugs have additional effects on the enzyme pyridoxal 5′-phosphate kinase (PLP-kinase), which regulates the availability of PLP in the brain. Consequently, GABA synthesis and glutamate decarboxylase activity are directly correlated with the availability of PLP, suggesting that PLP-kinase – a relatively unexplored enzyme – contributes to the regulation of GABA synthesis (TAPIA et al., 1969). Because of the risk of having convulsions, synthesis inhibition and measurement of GABA depletion is not a very useful way of studying functional changes in the GABA-ergic system. On the other hand, it has been used successfully to study the relation between GABA synthesis inhibition and the occurrence of convulsions (TAPIA, 1975).

1.2.8. GABA degradation

Several drugs known to interfere with GABA degradation (see TAPIA, 1975) act via PLP, which is an essential cofactor for glutamate decarboxylase and GABA-transaminase. They do not inhibit GABA-transaminase selectively, but have considerable effects on glutamate decarboxylase or PLP-kinase as well. Nevertheless,

some of these drugs may have a preferential effect on GABA-transaminase *in vivo* and they may increase the concentration of GABA in the brain, as is the case with aminooxyacetic acid (AOAA) in low doses (WOOD & PEESKER, 1972). Two drugs may inhibit GABA-transaminase selectively. Ethanolamine-O-sulphate is currently under investigation and it may inhibit GABA degradation selectively after intracerebral administration (FOWLER & JOHN, 1972; FOWLER, 1973). The catalytic inhibitor γ-vinyl-GABA has also been proposed as a selective inhibitor of GABA-transaminase (JUNG & METCALF, 1975; JUNG et al., 1976).

It is not certain whether inhibition of GABA-transaminase will result in a significant augmentation of GABA-mediated synaptic function. It has been suggested that GABA-transaminase is not involved in the control of GABA turnover. Moreover, inhibition of GABA-transaminase may increase GABA levels intracellularly without affecting the concentration of GABA at receptor sites. Certainly, the most interesting inhibitor of GABA metabolism is di-n-propylacetate (DPA). This drug was originally described by two groups as a rather weak inhibitor of GABA-transaminase (GODIN et al., 1969; SIMLER et al., 1973; FOWLER et al., 1975), but recent work of others as well as results described in this thesis suggest that DPA may inhibit SSA-dehydrogenase selectively (HARVEY et al., 1975; ANLEZARK et al., 1976).

1.2.9. Compartmentation of GABA

One of the main problems associated with almost every neurochemical or pharmacological study of the GABA-ergic system is its possible compartmentation in the CNS. It is not necessary to review all relevant data here since there are many books and reviews dealing with this subject (BERL, CLARKE & SCHNEIDER, 1975; BALÁZS & CREMER, 1973; BAXTER, 1976). However, many conclusions and concepts based on studies of intermediary metabolism are especially relevant for the neurotransmitter function of GABA. Baxter (1976) has defined compartmentation as follows: "Compartmentation refers to the presence in tissue of more than one pool or compartment. The term is applied to compounds which appear to be metabolized in tissue at several first-order rates simultaneously. This then is a kinetic and not a morphological criterion of compartmentation. By definition, each compartment of a compound has its own metabolic rate which distinguishes it from the same compound in another compartment. Also, by definition, such compartments are not in rapid equilibrium with each other; if they were, they would not be kinetically detectable."

The most simplified compartmented model of GABA metabolism is described in Fig. 1.2.5. and is based upon the assumption that GABA synthesis and GABA degradation take place in different compartments (VAN DEN BERG & GAR-

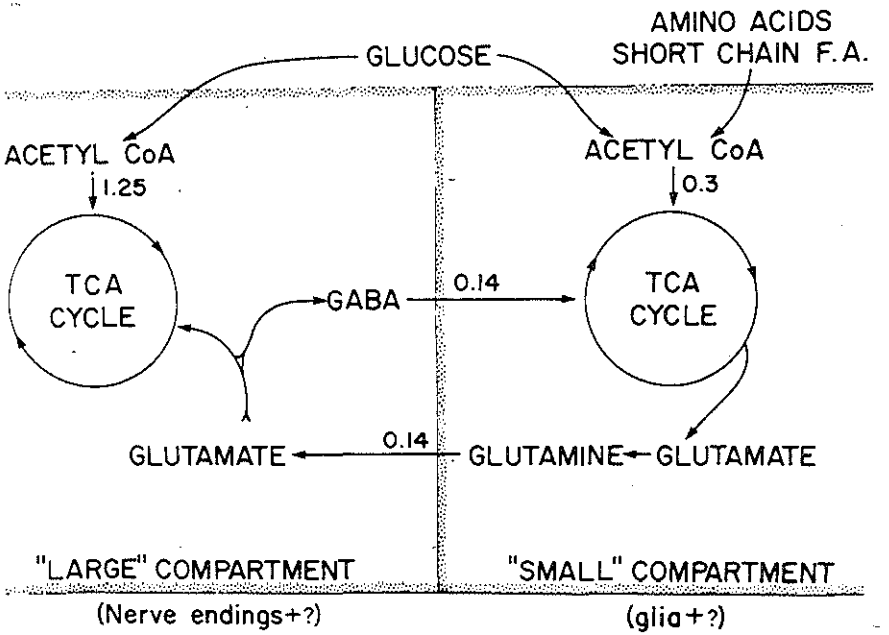


Fig. 1.2.5. Two-cycle model of mouse brain amino acid metabolism (BAXTER, 1976). The values indicated are fluxes in μmol/g wet wt/min. The two circles are tricarboxylic acid cycles.

FINKEL, 1971; VAN DEN BERG et al., 1975; BAXTER, 1976). In this model the results of compartmentation studies by VAN DEN BERG & GARFINKEL (1971) are combined with results of differential centrifugation studies demonstrating that glutamate decarboxylase is preferentially located in synaptosomal fractions, while nearly all GABA-transaminase is located in mitochondria of non-synaptic origin (SALGANICOFF & DE ROBERTIS, 1965; VAN KEMPEN et al., 1965; VAN DEN BERG et al., 1975). The result is a model locating GABA synthesis in nerve endings, from which it can be released into the synaptic cleft upon stimulation. It is then taken up into neuronal or glial elements, where it is degraded by GABA-transaminase and SSA-dehydrogenase. It must be recognised, however, that this model can only be a simple approximate of reality. For example, though the exclusive localization of glutamate decarboxylase in nerve endings has been questioned, no clear cut evidence against this hypothesis has been presented yet. However, both GABA-transaminase and SSA-dehydrogenase have been demonstrated in nerve ending fractions, glial cells and neuronal cell bodies (SALGANICOFF & DE ROBERTIS, 1965; SIMS & DAVIS, 1973; BUU & VAN GELDER, 1974).

1.2.10. GABA turnover

GABA turnover measurements using labelled glutamate or glucose as a precursor for GABA are difficult to interpret because glutamate is compartmented in the brain (VAN DEN BERG et al., 1975). It is probably not possible to separate these two, or possibly more, glutamate compartments and the interpretation of labelling data in terms of quantitative differences in fluxus or metabolic rates under different experimental conditions is difficult (BAXTER, 1976). The unknown relation between GABA and its precursor glutamate *in vivo* in relation to different functional pools of GABA, viz. nerve ending GABA or glial GABA, is responsible for these uncertainties. The suggestion that glutamine rather than glutamate may be the precursor of GABA (VAN DEN BERG & GARFINKEL, 1971) has found recent experimental proof (FONNUM, 1975b) by the demonstration that glutamine is the precursor for GABA in the substantia nigra.
Recently, ^{13}C-glucose has been used as a precursor for GABA to study the turnover of GABA *in vivo* under different pharmacological conditions (MARCO et al., 1976). Selective effects on regional GABA turnover were observed using different types of anti-psychotics. However, because glucose was used as the precursor of GABA it is not clear which GABA compartment is affected by the treatments. In fact, both glutamate compartments are labelled by glucose and may be affected by the treatments. Therefore, the model used by the authors to interpret their kinetic data, may be completely erroneous. COLLINS (1973) studied GABA turnover in different brain regions by measuring the disappearance rate of intraventricularly injected ^{3}H-GABA. Though of some interest because of the methodology of approach, these studies may be wrongly interpreted as the authors ignored the problems associated with the compartmentation of GABA (COLLINS, 1973; BAXTER, 1976). Another approach has been to measure the post-mortem synthesis of GABA after injection of labelled glutamate as a precursor. From these studies it appears that two GABA compartments have to be defined (PATEL et al., 1974). In these experiments it is assumed that no post-mortem synthesis of glutamate occurs, while the degradation of GABA is prevented by the abrupt decrease of NAD – the essential cofactor for SSA-dehydrogenase – after death.

1.2.11. Firing inhibition

The use of direct-acting GABA receptor stimulants and GABA receptor antagonists has long been restricted to electrophysiological studies using changes in cell-firing as the pharmacological response. Extensive reviews have been published reflecting an ongoing debate in the middle sixties between Curtis and co-workers and Krnjević and co-workers as to whether GABA might fulfill the role of *the*

Table 1.2.1.
GABA and some of its analogues (from KELLY & BEART, 1975)

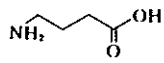
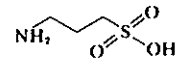
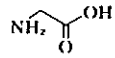
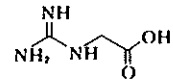
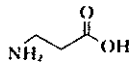
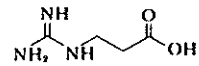
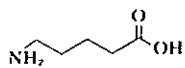
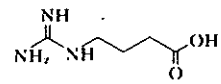
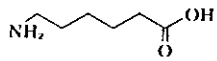
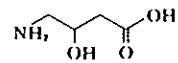
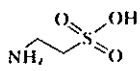
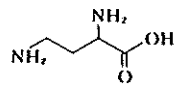
Compound	Structure	Compound	Structure
γ-Aminobutyric acid (GABA)		3-Aminopropanesulfonic acid	
Glycine		Guanidinoacetic acid	
β-Alanine		β-Guanidinopropionic acid	
δ-Aminovaleric acid		γ-Guanidinobutyric acid	
ε-Aminocaproic acid		γ-Amino-β-hydroxybutyric acid	
Taurine		2,4-Diaminobutyric acid	

Table 1.2.2.

Comparison of the activities of some GABA-like amino acids on several test preparations (from KELLY & BEART, 1975)

	Relative activity (GABA = 1)								
Amino acid	Rat ganglion (1)	Dog blood pressure (2)	Crayfish stretch receptor (3)	(4)	Crayfish neuromuscular junction (5)	(6)	Toad spinal cord (7)	Cat moto-neurons (8)	Cat cortical inhibition (9)
GABA	1	1	1	1	1	1	1	−−−	++++
Glycine	<0.0001	0	0.0008	0	0.00014	0	0.67	−−	++
β-Alanine	0.01	0.007	0.05	0.11	0.033	0.02	1.4	−−−	++++
δ-Aminovaleric acid	0.048	0.01	0.07	0.04	0.02	0.05	0.22	−−	++
ε-Aminocaproic acid	<0.0001	0	0.01	0.02	0.0005	<0.001		−	0
Taurine	0.001	0.001	0.005	0.004		<0.02	1	−−	0
3-Aminopropanesulfonic acid	3.4						3.3		
Guanidinoacetic acid	0.057		0.57	0.57	0.33		1	0	++++
β-Guanidinopropionic acid	0.12		0.71	1.23	0.5		0.17		++++
γ-Guanidinobutyric acid	0.0007		0.12	0.03	0.01		0	0	++
α-Amino-*n*-butyric acid	<0.0001	0				0		0	0
β-Amino-*n*-butyric acid	<0.0001				0.0003	<0.001	0	0	0
α-Aminoisobutyric acid	<0.0001	0	0.002	0.002				−	
γ-Amino-β-hydroxybutyric acid	0.27			0.14	0.5			−−	++++
2,4-Diaminobutyric acid	0.0012		0.005			<0.01	0.14	−	+
N-Methyl-GABA	0.005								

*Numbers in columns (1) to (7) give molar potencies as defined in the text: GABA = 1. In columns (8) and (9) relative activities are given in the arbitrary units employed by the original investigators (see references). In (8) the number of − signs is inversely related to the amount of current required to discharge an effective amount of drug iontophoretically; in (9) the number of + signs is proportional to the depression of evoked responses in the cerebellar cortex on topical application of the compounds in 0.1–1% solution. References: (1) Bowery and Brown (1974), (2) Stanton and Woodhouse (1960), (3) Edwards and Kuffler (1959), (4) McGeer *et al.* (1961), (5) Dudel (1965), (6) Robbins (1959), (7) Curtis *et al.* (1961), (8) Curtis and Watkins (1960), (9) Purpura *et al.* (1959).

inhibitory transmitter in the mammalian nervous system. Later on it appeared that glycine is the main inhibitor in the spinal cord, while GABA is more potent in the cerebral cortex (CURTIS & WATKINS, 1965; KRNJEVIC et al., 1966a, 1966b and 1966c; CURTIS & JOHNSTON, 1973). From these studies it is concluded that a straight-chained amino acid for having a GABA-like depressant action should obey the general formula:

$$NH_2(CH_2)_nX$$

where n = 2-4 and X is CO_2H, SO_2H or SO_3H. Some substitutions are allowed in the carbon chain (e.g. GABOB, γ-amino-β-hydroxybutyric acid), while a guanidino group together with shortening of the carbon chain to 1 or 2 is also possible. Some of these analogues and their physiological activities in several test preparations are shown in Tables 1 and 2. The naturally occurring GABA-like amino acids β-alanine, taurine, homohypotaurine, GABOB, imidazole-4-acetic acid and GABA itself may all function as inhibitory neurotransmitters in the brain with separate receptors (KELLY & BEART, 1975).

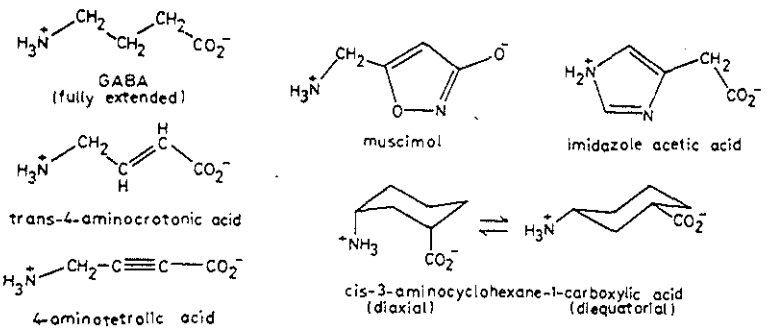
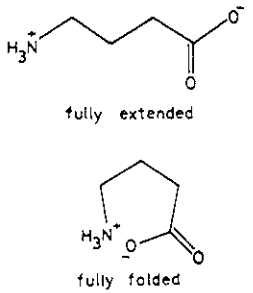


Fig. 1.2.6. Structures of GABA and some GABA analogues of restricted conformation. Only chair conformations of the cyclohexane derivatives are shown (from KELLY & BEART, 1975).

1.2.12. GABA agonists

GABA can exist in different conformations varying between the two extremes of fully extended and fully folded (Fig. 1.2.6.). Different conformations of GABA may be stabilized by proteins involved in different biochemical processes as GABA uptake and GABA binding to different neuronal elements (glial cells, neuronal cell body), biotransformation by GABA-transaminase and binding by more or less purified lipoproteins (GABA receptor). Some GABA analogues of



	N to O distance (Å) maximum	minimum
GABA	6.2	<1.5
trans-4-Aminocrotonic acid	6.1	4.9
cis-3-Aminocyclohexane-1-carboxylic acid	Diequatorial conformation	
	6.2	4.8
	Diaxial conformation	
	3.9	<1.5
4-Aminotetrolic acid	5.8	5.2

Fig. 1.2.7. Structures of fully extended and fully folded conformations of GABA. Interatomic distance of N and O for GABA and some conformationally restricted analogues were estimated by measurements of dreiding stereomodels (from BEART et al., 1972).

restricted conformation and with a strong depressant action are shown in Fig. 1.2.6, including the psychoactive isoxazole muscimol. Structure activity studies implicate the zwitterionic nature and the intramolecular distance between the two charged centres as essential factors (Fig. 1.2.7). The action of the conformationally restricted GABA analogues muscimol, 4-aminotetrolic acid, trans-4-aminocrotonic acid and imidazole-4-acetic acid are all antagonized by bicuculline, a specific GABA antagonist, suggesting that the GABA receptor prefers the extended conformation (JOHNSTON et al., 1968; CURTIS et al., 1971a and 1971b; BEART et al., 1971; BEART et al., 1972; KELLY & BEART, unpublished and cited by KELLY & BEART, 1975).

1.2.13. GABA binding

Several types of GABA binding can be distinguished in the rat CNS. The so-called sodium-dependent binding (binding in the presence of Na^+) probably represents the binding of GABA to uptake sites and it has many properties in common with GABA uptake. Three groups have succeeded in the isolation of the putative GABA receptor lipoprotein or GABA receptor lipoprotein containing membrane fraction (FISZER DE PLAZAS & DE ROBERTIS, 1975; PECK et al., 1976; YOUNG et al., 1976). The characteristics of these GABA-receptor fractions are quite different. The first group described a receptor with a K_D of 0.37 μM and a binding capacity of 0.7 pmol/mg protein, the second group reported on a fraction with 190 pmol/mg protein binding capacity and a K_D of 23 μM, while the third group obtained a fraction from the cerebral cortex with sodium-independent binding with a K_D for GABA of 30 μM and a binding

capacity of 0.6 pmol/mg protein in retina and rat brain tissue; at the same time "low affinity" binding was observed with a K_D of 220 nM and a binding capacity of 1.6 pmol/mg protein (ENNA & SNYDER, 1976). This high affinity binding was only observed in the presence of triton X-100 – a membrane detergent – and could be antagonized stereoselectively by bicuculline with a K_i of 0.8 μM. Picrotoxine, another GABA antagonist, was not effective in these studies. The GABA analogues 3-aminopropane sulfonic acid, muscimol and imidazole acetic acid were also effective in displacing radioactive GABA from its binding site. Bicuculline is a competitive antagonist in all systems used for GABA binding.

Table 1.2.3.
Substrate specificity of the GABA receptor

Compound	ID_{50} (μM)			
	Sodium-dependent GABA binding	Synaptosomal GABA uptake	Sodium-independent GABA binding	% GABA-like neurophysiologic activity[a]
GABA	1.2	10	0.37	100
3-Aminopropanesulfonic acid	160	1,400	0.25	130–150
Imidazoleacetic acid	100	>1,000	0.24	90–100
1-Methylimidazoleacetic acid	–	>1,000	>1,000	0
3-Hydroxy GABA	–	100	1.0	50–70
β-Alanine	35	55,000	80	30–50
2,4-Diaminobutyric acid	540	260	>1,000	5–10
p-Aminophenylmercuric acetate	11	2.6	>1,000	0
Chlorpromazine	21	12	160	0
d-Tubocurarine	860	7,500	38	0
Bicuculline	130	>1,000	4	0
Strychnine	–	100	100	0

(This table is taken from YOUNG et al., 1976)

The first group has compared sodium-dependent and sodium-independent binding and observed marked differences in the characteristics of both systems (Table 1.2.3). The effect of many GABA analogues runs roughly in parallel with their known physiological effect as indicated above. The authors conclude that the sodium-dependent binding presumably represents glial uptake, since it was rather sensitive for the supposed selective inhibitor of glial GABA uptake β-alanine (SCHON & KELLY, 1974). Conformationally restricted GABA analogues have not yet been used in these binding studies, but may prove very useful since sodium-dependent and sodium-independent binding may prefer different conformations of GABA.

1.2.14. GABA antagonists

At present only four pharmacologically useful GABA antagonists can be distinguished, viz. bicuculline, picrotoxine, d-tubocurarine and penicillin (Fig. 1.2.8). All four drugs contain one or more "zwitterion" structures with two oppositely charged groups 5 to 6 Å apart (KELLY & BEART, 1975). However, their mode of action is still uncertain. Picrotoxine, for example, does not affect GABA binding in a preparation where bicuculline is active (FISZER DE PLAZAS & DE ROBERTIS, 1975; PECK et al., 1976). In the preparation of YOUNG et al. (1976) d-tubocurarine and bicuculline were both active as antagonists of sodium-independent GABA binding, while picrotoxine was not. Neurophysiologically, picrotoxine is thought to impair chloride conductance changes associated with the action of GABA on the stretch receptor of the crayfish rather than to compete with GABA for the recognition site (TAKEUCHI & TAKEUCHI, 1969). This may account for the inability of picrotoxine to compete successfully with GABA for the binding site.

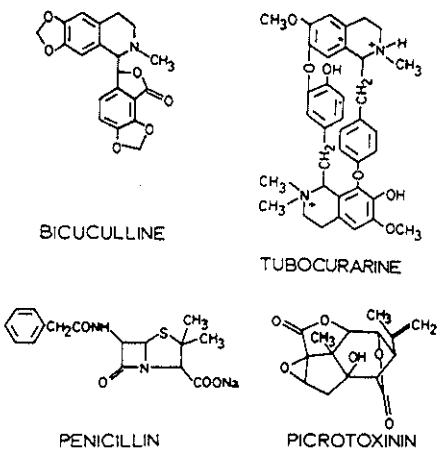


Fig. 1.2.8. Structures of some GABA antagonists (from KELLY & BEART, 1975).

1.2.15. GABA uptake

It seems rather unlikely that the physiological action of GABA in the synaptic cleft can be terminated by GABA degradation. GABA metabolism is located intracellularly and requires the presence of PLP and 2-oxoglutaric acid, which are also localized inside neuronal elements. Instead, the uptake system of GABA, present in nervous tissue, can accumulate extracellularly ^{3}H-GABA very rapidly

resulting in tissue-medium ratios of 100 to 1. This very rapid uptake process may clear GABA from extracellular space of brain slices within less than two seconds (IVERSEN, 1971). Many studies in several preparations (slices of brain regions, isolated organs, e.g. ganglia, subcellular structures like synaptosomes, particular cells like cell bodies and glial cells) have shown that GABA transport is mediated via a temperature dependent, saturable process, which is dependent on the presence of sodium ions. As is the case for other neurotransmitters a high and a low affinity uptake system could be distinguished for GABA with affinity constants of 0.1-10 μM and 1 mM respectively (BENNET et al., 1974). The high affinity system is thought to prevent GABA from accumulation in the synaptic cleft.

1.2.16. Uptake inhibitors

Extensive studies appeared to elucidate the structural requirements of GABA uptake inhibitors (for reviews, see: IVERSEN & KELLY, 1975; TAPIA, 1975; MARTIN, 1976). These studies demonstrated the presence of yet another complicating factor, since synaptosomes as well as purified glial preparations were capable of high affinity transport of labelled GABA (SCHON & KELLY, 1974). Studies of HAMBERGER and co-workers showed that synaptosomal preparations may contain as much as 40% of "contamination" with vesicles of glial origin (HENN et al., 1976). Using these types of preparations, however, specific inhibitors could be detected for glial or synaptosomal uptake (SCHON & KELLY, 1974). These findings, relating 2,4-diaminobutyric acid (DABA) to synaptosomal or neuronal uptake, but β-alanine to glial uptake, were not confirmed by others (SNODGRASS et al., 1973, SELLSTRÖM & HAMBERGER, 1975). These latter studied were performed in bulk preparations of glial and neuronal cells whose structural integrity has been recently criticized (BALÁZS, 1976). By using labelled DABA, IVERSEN & KELLY (1975) were able to demonstrate high affinity uptake of this GABA analogue in synaptosomes, but not in isolated rat sensory ganglia. On the other hand, labelled β-alanine was exclusively recovered in glial preparations from sensory ganglia and cerebral cortex slices. They suggest the use of labelled β-alanine and DABA as selective inhibitors of glial and neuronal GABA uptake respectively, to allow a better functional distinction to be made between these two uptake systems (IVERSEN & KELLY, 1975). The usefulness of β-alanine and DABA as selective inhibitors of glial and neuronal uptake, respectively, has been demonstrated recently in two *in vivo* studies (SUTTON & SIMMONDS, 1974; HO et al., 1976).

1.2.17. Homoexchange of GABA

Though the concept for uptake described in the foregoing part has found widespread acceptance, recent findings on GABA uptake have resulted in serious criticism of this hypothesis (LEVI & RAITERI, 1974; IVERSEN, 1975; LEVI & RAITERI, 1975; RAITERI et al., 1975). Levi et al., but not Iversen et al., believe that homoexchange of labelled GABA with endogeneous GABA might account for a considerable part of the initial rates of GABA accumulation of so-called high affinity uptake of labelled GABA. They suggest that, even if no high affinity system for GABA uptake may exist, a low affinity system for GABA uptake with a K_m of 1 mM may be effective enough to account for synaptic inactivation (LEVI & RAITERI, 1975). Influx and efflux of GABA has similar sodium dependency, temperature sensitivity and kinetic properties suggesting the involvement of carrier-mediated exchange diffusion (SIMON et al., 1974; STORM-MATHISEN et al., 1976). Net efflux could be demonstrated by these authors in synaptosomal preparations, which may possibly represent sodium-independent efflux (MARTIN, 1976).

1.2.18. Glial uptake

The glial high affinity uptake system may be involved in the proposed cycle of carbon transfer from neuronal elements into glial cells (VAN DEN BERG, 1973; BALÁZS et al., 1973a). However, not all glial cells are located near to GABA-releasing nerve endings, though they possess the machinery to metabolize GABA. Therefore, in glial cells GABA may have a function not directly related to its function as an inhibitory neurotransmitter (IVERSEN & KELLY, 1975).

1.2.19. Extra-cerebral GABA

Moderate rates of GABA metabolism have been found in the kidney (VAN GELDER, 1965) and in other peripheral organs (ZACHMANN et al., 1966; WHELAN et al., 1969; HABER et al., 1970a, b and c; WU et al., 1974; DRUMMOND & PHILLIPS, 1974). The report of very high levels of GABA in certain areas of the pancreas and the reports mentioned above suggest than inhibition of neuronal firing in the CNS and carbon transfer are not the only functions of GABA in the living organism (OKADA et al., 1976).

1.3. GABA IN REGIONAL NERVOUS TISSUE

In this chapter the functions of GABA and glycine as inhibitory transmitters in different brain regions and spinal cord are discussed. As the function and regulation of GABA may vary considerably among different areas in the brain and spinal cord, it is intended to summarize the available evidence for specialized functions of GABA in these regions of the CNS.

1.3.1. Comparison of the effects of GABA and glycine in spinal cord

1.3.1.1. Localization of GABA and glycine in grey and white matter

Dissection of the spinal cord into several sections containing white and grey matter revealed that the concentration of glycine is higher in grey matter than in white matter, while similar results are obtained for GABA or other amino acids (Table 1.3.1). However, the rostrocaudal distribution for glycine is very indicative of a special function for glycine in the spinal cord. The opposite is observed for GABA, suggesting that both neutral amino acids have different functions in the spinal cord. Immunohistochemical techniques demonstrate a close relationship between the localization of the primary afferent depolarization and GABA, since antibodies against glutamate decarboxylase as well as the primary afferent depolarization are preferably associated with substantia gelatinosa Rolandi (BARBER & SAITO, 1976; WOOD et al., 1976). Similar results have been obtained by using quantitative histochemistry to measure GABA in reference to presynaptic inhibition (MIYATA & OTSUKA, 1975). Also, these experiments indicate that cauterization of blood vessels supplying the dorsal horns in cats (unilaterally) decreases GABA in the dorsal part of the dorsal horn (OTSUKA & KONISHI, 1976) as indicated in Fig. 1.3.1.

Table 1.3.1.
Distribution of GABA, glycine and other amino acids in cat spinal cord*

	GABA	Gly	Asp	Glu	Gln
Dorsal gray	2.23	5.65	2.05	6.48	5.30
Ventral gray	1.07	7.08	3.06	5.39	5.35
Dorsal white	0.43	3.04	1.11	4.80	3.59
Ventral white	0.44	4.39	1.29	3.89	3.81
Dorsal root	0.06	0.64	1.50	3.80	1.61
Ventral root	0.08	0.64	1.24	2.20	1.53

* Values are in μmol/g (from RYALL, 1975).

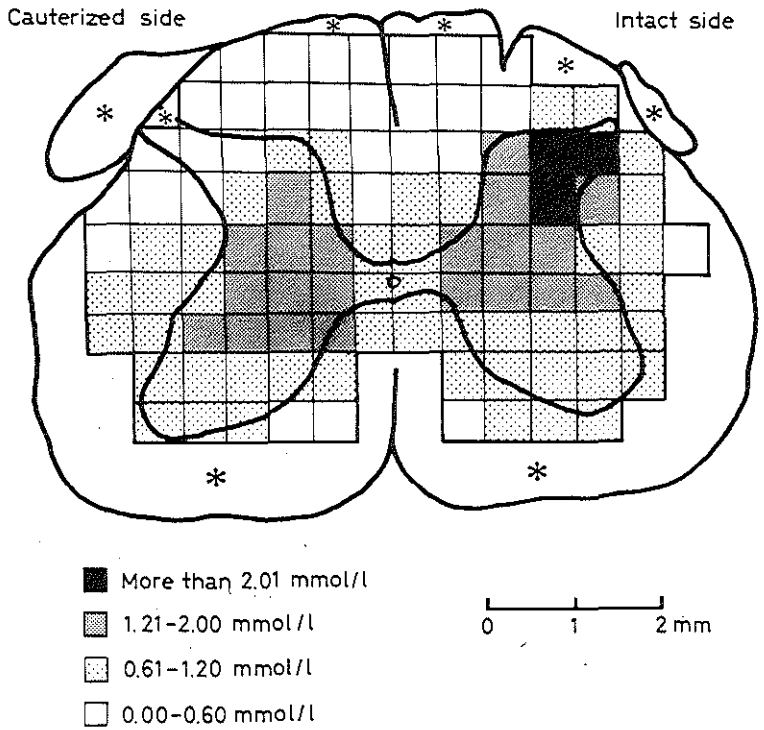


Fig. 1.3.1. GABA distribution in L6 segment of cat spinal cord 9 days after unilateral cauterization of blood vessels supplying the dorsal horn (from MIYATA & OTSUKA, 1975).

1.3.1.2. Effects of glycine on postsynaptic cells and presynaptic terminals

Motoneurons in spinal cord can be hyperpolarized by iontophoretically administered glycine or GABA (WERMAN et al., 1968; CURTIS et al., 1968), though glycine is more effective than GABA. The membrane conductance of motoneurons is increased by glycine, while it also produces a hyperpolarization of the membrane with an equilibrium potential that is almost identical to the inhibitory postsynaptic potential (IPSP). Similar results have been obtained for interneurons like Renshaw cells. Strychnine suppresses the inhibitory action of glycine on motoneurons, interneurons, on Renshaw cells and on sacral parasympathetic neurons (LARSON, 1969; DAVIDOFF et al., 1969; RYALL et al., 1972; DE GROAT, 1970b) and is currently considered as a specific antagonist of the inhibitory action of glycine on cell-firing. The finding that strychnine has

a potent action on postsynaptic inhibition strongly favours the idea that glycine is a major mediator of postsynaptic inhibition in spinal cord (RYALL, 1975). On the other hand, strychnine is not effective against presynaptic inhibition nor does it affect the inhibitory effect of GABA on presynaptic terminals. This suggests that glycine is not the mediator of presynaptic inhibition in the spinal cord.

1.3.1.3. Effects of GABA on postsynaptic cells and presynaptic terminals

Presynaptic inhibition is thought to be a phenomenon that acts by reducing the release of an excitory transmitter from presynaptic terminals, but the same mechanism might also result in disinhibition by preventing the release of an inhibitory transmitter. This presynaptic inhibition is mediated by axo-axonic synaptic contacts, i.e. presynaptic inhibitory terminals ending on the terminals of afferent fibers. Though the evidence is not complete a role of GABA-ergic synapses in the so-called P-wave, the dorsal root potential (DRP), the dorsal root reflex (DRR) and the primary afferent depolarization (PAD) is evident for the following reasons. Firstly, GABA and its like-acting congeners 3-amino-n-valeric acid, β-alanine or 3-aminopropanesulfonic acid can produce many of the effects of presynaptic inhibition, like a depression of the DRP and to a lesser extent of the P-wave (ECCLES et al., 1963) (with a slow recovery, suggesting the occurrence of "prolonged inhibition" which is characteristic of presynaptic inhibition) and an increase of DRP, while a change in the excitability state of the terminals has not been demonstrated. It is concluded by SCHMIDT (1971), that locally applied GABA may reduce the DRP, while having additional effects at other sites in the PAD pathway. A summary of many conflicting data is given by RYALL (1975) who concluded that hyperpolarization may occur with low GABA concentrations while a higher concentration may result in release of K^+ into the extracellular space. This results in the so-called remote inhibiton as suggested from experiments of TEBĒCIS & PHILLIS (1969) and CURTIS & RYALL (1966). Similar results are obtained in the cuneate nucleus, which may have a somewhat intermediate position between spinal and supraspinal mechanisms (GALINDO, 1969).

Additional support for a role of GABA in presynaptic inhibition comes from experiments with the GABA antagonists picrotoxine and bicuculline. Picrotoxine produces convulsions via a selective depression of presynaptic inhibition in the spinal cord (ECCLES et al., 1963; SCHMIDT, 1963), it blocks the GABA-induced depolarization of sensory root ganglia *in vivo* and in tissue culture (DE GROAT et al., 1972; OBATA, 1972). However, picrotoxine has not been considered to be very reliable as a GABA antagonist and the search for a better antagonist produced the more selectively acting GABA antagonist, bicuculline (CURTIS et al., 1971a). Bicuculline is ineffective against direct, recurrent or

Table 1.3.2.

Distribution of glutamate-decarboxylase in brain nuclei of the rat

Brain nuclei	*GAD activity*
Diencephalon *(continued)*	
Hypothalamus	
N. preopticus lateralis	560 ± 20
N. paraventricularis	409 ± 36
N. arcuatus	285 ± 38
N. ventromedialis	390 ± 34
N. dorsomedialis	556 ± 61
Median eminence	109 ± 15
Medial forebrain bundle	463 ± 40
Mamillary bodies	460 ± 17
Subthalamus-methalamus-epithalamus	
Corpus geniculatum laterale	341 ± 27
Corpus geniculatum mediale	331 ± 32
Zona incerta	325 ± 35
Habenula	472 ± 31
Mesencephalon	
Substantia nigra	
Pars compacta	484 ± 20
Pars reticularis	1110 ± 30
Colliculi	
Inferior colliculus	636 ± 72
Superior colliculus	743 ± 112
Substantia grisea centralis	574 ± 67
Nucleus ruber	332 ± 36
Nucleus interpeduncularis	293 ± 37
Rhombencephalon	
Pons	
Nuclei ponti	112 ± 11
Tegmentum ponti	465 ± 100
Nucleus trapezoidus	133 ± 20
Cerebellum	
Cortex	230 ± 16
Nuclei	251 ± 37
Reticular formation	153 ± 8
Nucleus gracilis	199 ± 20
Nucleus cuneatus	246 ± 28
Inferior olive	181 ± 86
Superior olive	154 ± 19
Cranial nerve nuclei	
N. tractus spinalis trigemini	252 ± 12
N. nervi facialis	190 ± 13
N. cochlear	165 ± 24
N. vestibularis spinalis	246 ± 15
N. vestibularis medialis	328 ± 33
N. vestibularis lateralis	216 ± 11
N. vestibularis superior	265 ± 21

Brain nuclei	*GAD activity*
Whole brain homogenate	230 ± 16
Telencephalon	
Frontal cortex	325 ± 20
Parietal cortex	323 ± 25
Limbic cortex	
Gyrus dentatus	413 ± 54
Cingulate cortex	380 ± 12
Piriform cortex	220 ± 13
Entorhinal cortex	225 ± 34
Hippocampus	409 ± 40
Rostral limbic system	
Olfactory tubercles	526 ± 28
Olfactory bulb	630 ± 45
N. tractus olfactorius lateralis	257 ± 18
N. tractus diagonalis	665 ± 68
N. accumbens	574 ± 70
N. interstitialis striae medullaris	506 ± 35
Septum	
N. septalis dorsalis	456 ± 35
N. septalis medialis	332 ± 30
N. septalis lateralis	496 ± 35
N. septalis fimbrialis	250 ± 13
Amygdaloid nuclei	
N. amygdaloideus corticalis	349 ± 33
N. amygdaloideus medialis	310 ± 15
N. amygdaloideus basalis	341 ± 34
N. amygdaloideus lateralis	333 ± 38
N. amygdaloideus centralis	422 ± 40
Basal ganglia	
Caudate putamen	270 ± 10
Globus pallidus	461 ± 40
Claustrum	426 ± 15
Circumventricular organs	
Area postrema	131 ± 18
Subfornical organ	98 ± 10
Subcommissural organ	245 ± 25
Organum vasculosum lamina terminalis	124 ± 15
Diencephalon	
Thalamus	
N. reticularis thalamus	186 ± 13
N. anterioventralis thalamus	234 ± 25
N. ventralis thalamus	226 ± 26
N. posterior thalamus	497 ± 26

(from TAPPAZ et al., 1976)

Golgi inhibitions, which are sensitive to strychnine, but it inhibits the prolonged spinal inhibition as does picrotoxine. Furthermore, it blocks GABA-induced depolarization of dorsal root ganglia and similarly, the depolarization of primary afferents (DE GROAT et al., 1972; BARKER & NICOLL, 1973). As a result it has been finally suggested that GABA is the transmitter at synapses that are insensitive to strychnine, but sensitive to bicuculline or picrotoxine (CURTIS et al., 1971a; LEVY et al., 1971; HUFFMAN & McFADIN, 1972). However, the

picture may be less clear and straightforward than suggested here (RYALL, 1975).

1.3.2. Comparison of the effects of GABA and glycine on supraspinal regions

1.3.2.1. The distribution of glutamate decarboxylase in brain nuclei of the rat

This chapter is started with the very valuable study of TAPPAZ and co-workers (1976) which has resulted in a detailed map of the distribution of GABA synthesis in most brain nuclei of the rat. This study represents the first evidence for an uneven distribution of the GABA-ergic system in many brain nuclei, including the hypothalamus and the thalamus.

1.3.2.2. Inhibition in the cerebral cortex

Levels of GABA are not particularly high in cortical grey matter but they exceed those in white matter (CURTIS & JOHNSTON, 1973). GABA is high in single Betz cells where it is 2.5 mM in the cat (OTSUKA et al., 1971). Additionally, GABA is concentrated in particular layers of the cerebral cortex. Similarly, glutamate decarboxylase as well as GABA-transaminase are also unevenly distributed over functionally different cortical layers (ALBERS & BRADY, 1959; SALVADOR & ALBERS, 1959). Injection of labelled GABA into parietal cortex results in preferential accumulation by the stellate cells of layers II and III (HÖKFELT & LJUNGDAHL, 1972). Firing inhibition of cortical neurons by GABA is associated with hyperpolarization of the membrane and consequently with an increase of conductance by changes in the permeability of chloride ion (DREIFUSS et al., 1969). In general, a close resemblance exists between the postsynaptic action of GABA on cortical neurones and the natural occurring inhibitory transmitter released upon the cell-bodies of cortical pyramidal cells (CURTIS & JOHNSTON, 1973; KELLY & BEART, 1975). Bicuculline, but not strychnine, the selective antagonist of glycine, antagonizes the inhibitory action of GABA or like acting congeners on cat cortical neurones, though such a selectivity has not been demonstrated in rat cortical neurons. Similar results have been obtained with picrotoxine, which also lacks selectivity between glycine and GABA in the rat, but not in the cat. Therefore, a portion of the stellate cells of cerebral cortex may be inhibitory, releasing GABA at axo-somatic synapses on pyrimidal cells (CURTIS & FELIX, 1971), while the importance of glycine as a cortical inhibitory transmitter is minimal.
Though bicuculline is a very valuable GABA antagonist in other supraspinal regions (see further) serious doubts exist concerning its GABA blocking capacity

in cerebral cortex. Instead, d-tubocurarine has been shown to be a very reliable GABA antagonist in this brain region (HILL et al., 1973). Though the low solubility of bicuculline is a serious problem in iontophoretic studies, control experiments indicate that bicuculline is released but appears inactive on many neurons in the cerebral cortex. Two possibilities have been suggested to account for the inability of bicuculline to act as a reliable GABA antagonist in the cerebral cortex. The first is that GABA receptors in the cerebral cortex are unique and not similar to those at sites where bicuculline is active. The second possibility is that bicuculline is only effective in relatively simply organized neuronal networks. In more complex networks it is possible that an inhibitory neuron releasing GABA on the neuron under study, is itself also under the influence of an inhibitory transmitter that is blocked by the antagonist being studied. The outcome will then be unpredictable. Using well-defined pathways CURTIS & FELIX (1971) could stimulate the inhibitory neuron by the suspected activatory mechanism under which influence the inhibitory neuron is acting *in vivo*. As a result a very easy blockade by bicuculline is obtained. Therefore, the complexity of the neuronal connections in the cerebral cortex may be responsible for the relative ineffectiveness of bicuculline in operating as a recognized antagonist in this particular brain region.

1.3.2.3. Inhibition in cerebellum

Many investigators have stressed the importance of studying the cerebellum for the following reasons. The anatomical organization of the cerebellum is well defined; there are only five cell types, which are organized in different layers. These cell-types are the Purkinje cell, the basket cell, the superficial stellate cell, the Golgi cell and the granule cell, which are interrelated by way of three types of fibers: the mossy fibers, the climbing fibers and the parallel fibers (ITO, 1976). Presumably, GABA is the principle inhibitory transmitter of the Purkinje cell mediating the inhibition of neurons in the dorsal Deiters nucleus via Purkinje cell terminals (FONNUM & WALBERG, 1973; OBATA, 1976). COSTA et al. (1976) have correlated changes in the GABA-ergic system with c-GMP changes utilizing a mutant mice strain devoid of the GABA releasing Purkinje cells, the principle output of the cerebellum. BALÁZS and co-workers have recently succeeded in isolating a fraction of cell bodies from the cerebellum while preserving a high degree of morphological integrity (BALÁZS et al., 1975; BALÁZS, 1975; HAJÓS & WILKIN, 1975; WILKIN et al., 1975; WILSON et al., 1975; BALÁZS, 1976).
GABA-transaminase and glutamate decarboxylase are unevenly distributed in cerebellar layers (SALVADOR & ALBERS, 1959; STORM-MATHISEN, 1976). Using immunological techniques it is demonstrated that all four intrinsic inhib-

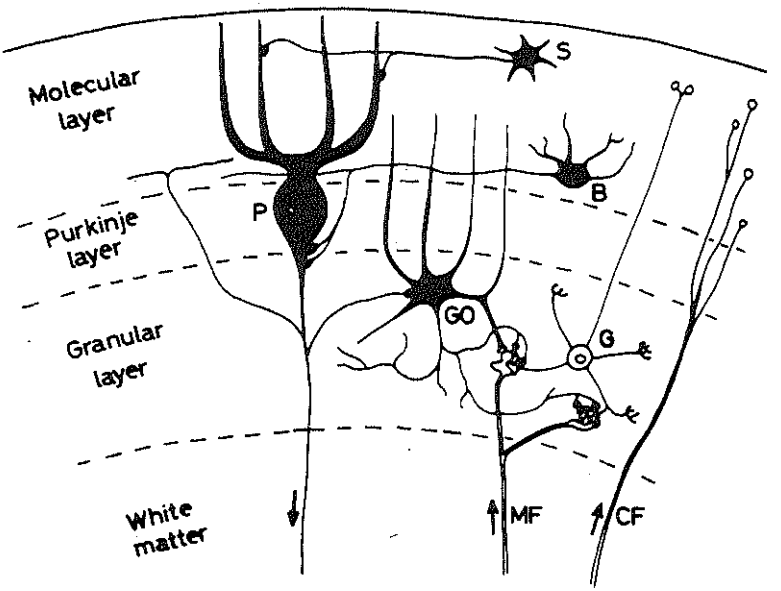


Fig. 1.3.2. The organization of cerebellar cells and their efferent and afferent connections (from STORM-MATHISEN, 1976).

itory neurons in the cerebellum are enriched in glutamate decarboxylase (BARBER & SAITO, 1976). Immunocytochemical techniques in combination with electronmicroscopic examination demonstrate that glutamate decarboxylase is associated with terminals derived from these four – presumably GABA-ergic – inhibitory neurons (WOOD et al., 1976).

In the cerebellum, the firing rate of Purkinje cells can be depressed most effectively by GABA, which causes hyperpolarization and consequently increases chloride ion conductivity. The inhibition of Purkinje cells following stimulation of cerebellar basket and stellate cells is not affected by strychnine, but is blocked by bicuculline and relatively high doses of picrotoxine (CURTIS & JOHNSTON, 1973). Thus an inhibitory action of four cerebellar inhibitory neurons using GABA as their transmitter, seems very likely. The cerebellum is therefore a unique structure for the study of the mechanisms involved in the inhibitory action of GABA (BALAZS, 1976). Inhibitory effects are observed with glycine in the cerebellum, but the potency ratio as compared to GABA is low and the inhibitory action of iontophoretically applied glycine is not antagonized by iontophoretically applied strychnine or intravenous strychnine (ANDERSEN et al., 1963; CRAWFORD et al., 1963; CURTIS & FELIX, 1971). The relative

ease with which the basket cell inhibition of the firing rate of Purkinje cells is antagonized by bicuculline again illustrates that inhibition antagonism is easy to demonstrate in relatively simple networks such as in cerebellum.

1.3.2.4. Inhibition in the thalamus

The thalamic nuclei have not been studied extensively in relation to the distribution of GABA and the activity of glutamate decarboxylase of GABA degrading enzymes. Lesion experiments indicate that GABA-ergic neurons are predominantly intrinsic (UTLEY, 1963; MARGOLIS et al., 1968). Measurement of glutamate decarboxylase and GABA degrading enzymes indicate an assymmetrical distribution, as is demonstrated by the high glutamate decarboxylase activity reported for N. posterior thalamus (TAPPAZ et al., 1976, see Table 1.3.2; FAHN, 1976). Presumably, GABA is the post-synaptic inhibitor of thalamic neurons released at the terminals of thalamic interneurons excited by stimulation of thalamo-cortical relay neurons. The prolonged inhibition of thalamic neurons observed after stimulation of afferent pathways is blocked by bicuculline, but not strychnine (CURTIS & JOHNSTON, 1973). According to ITO (1976), the thalamus contains Golgi type II GABA neurons which exhibit three forms of inhibition: post-synaptic inhibition exerted on thalamocortical relay-cells (feed forward inhibition), post-synaptic inhibition of thalamo-cortical axon collateral fed backwards to the relay-cells (feedback inhibition) and presynaptic inhibition among the lemniscal fibers. These GABA neurons may have two functions. Firstly, they may produce sharp focusing of excitation via lateral inhibition, and secondly, they may produce synchronized discharges (inhibitory phasing) involved in the production of brain waves (ANDERSEN et al., 1964).

1.3.2.5. Inhibition in the hypothalamus

The hypothalamus is known as the centre of the autonomic system. It contains large amounts of GABA and has been studied extensively in this respect (KURIYAMA & KIMURA, 1976). GABA and glutamate decarboxylase are unevenly distributed, but this does not coincide with certain particular nuclei. However, consistently, the highest levels are observed in the lateral hypothalamic area (LHA), while intermediate levels are observed in the anterior hypothalamus and the ventromedial nucleus of hypothalamus. Their experiments suggest that the content of GABA in the LHA is modified by changes in blood sugar, suggesting that the inhibitory amygdalafugal or pallidofugal fibers terminating in the LHA, may be GABA-ergic in nature. Glycine and GABA inhibit neurons in supraoptic paraventricular nuclei and their actions can be blocked by strychnine and bicu-

culline, respectively (CURTIS & JOHNSTON, 1973). Recent experiments with picrotoxine and bicuculline indicate the involvement of GABA-ergic mechanisms in the phenomenon of lateral hypothalamic self-stimulation (KENT & FEDINETS, 1976).

1.3.2.6. Inhibition in the basal ganglia

The concentration of GABA and the activity of glutamate decarboxylase are highest in substantia nigra and globus pallidus, while the caudate nucleus and putamen have much lower levels in the brain (FAHN, 1976). Considering that a high concentration of GABA is indicative of a dense projection of inhibitory neurons, the conclusion that GABA-ergic neurons massively project to substantia nigra and globus pallidus is justified. It is, however, far from clear where these GABA-ergic projections originate. Therefore, a discussion about the precise origin of GABA-ergic projections in substantia nigra and globus pallidus will follow.

Substantia nigra

GABA is unevenly distributed throughout the substantia nigra. A detailed study of KANAZAWA demonstrates highest concentrations in substantia nigra pars reticulata for GABA and glutamate decarboxylase (KANAZAWA et al., 1973; KANAZAWA & TOYOKURA, 1975). Whether the uneven distribution of GABA concentration reflects the *in vivo* situation, or originates from post-mortem synthesis of GABA from glutamate is of considerable importance for evaluating the reliability of GABA measurements in relation to GABA-ergic function. It has been demonstrated recently that the uneven distribution of GABA in substantia nigra can be completely absent after inhibition of the post-mortem synthesis of GABA using a microwave apparatus (TAPPAZ et al., 1977). FONNUM (1975a) has observed that in normal animals the main glutamate decarboxylase activity is confined to the central and medial parts of the pars reticulata and the lateral and central parts of the pars compacta. The medial part of pars compacta, primarly containing cell bodies, contains about 70% of the levels observed in the remaining parts of pars compacta.
Two opinions have been presented about the origin of the striato-nigral fibers. FONNUM et al. (1974) and KIM et al. (1971) favour the existence of a caudate-putamen source of these projections, probably with collaterals to the globus pallidus as also suggested recently by others (KANAZAWA & TOYOKURA, 1974). However, McGEER and co-workers conclude that these projections are pallidal-derived, since they have observed no change in glutamate decarboxylase

activity in substantia nigra after hemitranssection at the level of the ventromedial hypothalamus, i.e. anterior to the globus pallidus (McGEER et al., 1971; HATTORI et al., 1973). Analyses of the topographical organization of the striatonigral projections reveal that the fibers pass through the capsula interna, globus pallidus and nucleus entopeduncularis (in the cat) while projecting in the medial (for caudato-nigral) or lateral (for putamino-nigral) parts of the substantia nigra pars reticulata (FONNUM, 1975). Lesions at the level of the subthalamic nucleus result in a 70-90% loss of glutamate decarboxylase activity from substantia nigra. Because 80-85% of this enzyme seems to be particulate, this suggests that a very large part of the GABA-ergic input from striatum is lesioned by this procedure (HATTORI et al., 1973; KATAOKA et al., 1974; STORM-MATHISEN, 1975). Because large lesions – sometimes performed by suction of large parts of the caudate-putamen – in striatum do not produce such massive changes in nigral glutamate decarboxylase, some substantial GABA-ergic projection from globus pallidus is likely. The studies of FONNUM (1975) and KATAOKA et al. (1974), however, exclude the possibility of an exclusive projection from globus pallidus as suggested by McGEER et al. (1971). Since caudate lesions also produce changes in globus pallidus, some caudato-pallidal projection may exist (KANAZAWA & TOYOKURA, 1974). This confirms earlier electro-physiological results demonstrating that this caudato-pallidal projection is probably a collateral of the striato-nigral projection (YOSHIDA et al., 1972; YOSHIDA, 1974).

Uptake experiments with GABA using tissue derived from substantia nigra of rats with transsection of the striato-nigral pathway demonstrate a decrease of uptake to 30-40% of control values within 7 days after lesioning. At that time the remaining glutamate decarboxylase activity is only 10% of control values (STORM-MATHISEN, 1975). This suggests that 70% of the uptake in substantia nigra derived synaptosomes is affected by the lesion. This is also in accordance with autoradiographic studies demonstrating that nerve terminals are relatively less labelled after lesions using incubation with ^{3}H-GABA (HATTORI et al., 1973).

Electrophysiological evidence suggests that the inhibitory action of striato-nigral fibers on dopamine cell bodies in substantia nigra is mediated by GABA, since picrotoxine, but not strychnine, antagonizes the observed monosynaptic inhibition obtained after stimulation of striatum (YOSHIDA & PRECHT, 1971: PRECHT & YOSHIDA, 1971; OBATA & YOSHIDA, 1973). Similarly, it appears that iontophoretical application of GABA on nigral cells causes inhibition of cell firing, while iontophoretically applied glycine is not active (CROSSMAN et al., 1973; FELTZ, 1971). A detailed study of the structural synaptic organization of the substantia nigra has revealed the presence of 6 types of nerve endings. Type I, characterized by numerous densely packed empty synaptic vesicles varying in form from round to elliptical and elongated, is presumably GABA-ergic (HAJDU

et al., 1973; BAK et al., 1975). Following destruction of the striatum of the rat or cat, this type of nerve ending underwent degeneration. The GABA-ergic nature of these nerve endings has been confirmed in a subsequent autoradiographic study using the injection of ^{3}H-GABA (BAK et al., 1975). Numerous labelled contacts are observed with dendrites and nigral (presumably dopaminergic) cell bodies. Injection of ^{3}H-GABA into the striatum does not result in the arrival of labelled material in substantia nigra, but the injection of ^{3}H-GABA into globus pallidus is very effective. It produces labelling in symmetrical nerve endings contacting with presumed dopaminergic cell bodies as demonstrated by degeneration studies using 6-hydroxy-dopamine (McGEER et al., 1974).

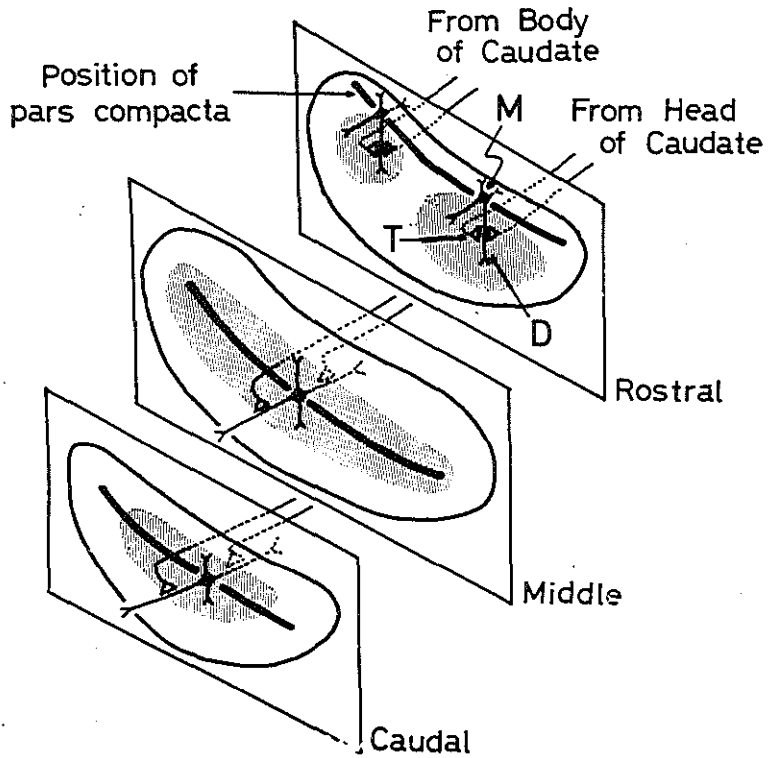


Fig. 1.3.3. Topographic distribution of GABA and glutamate decarboxylase in human substantia nigra. M, melanin-rich cell body; D, dendrite; T, GABA-containing nerve terminal; shaded area, GABA-rich area (from KANAZAWA & TOYOKURA, 1975).

Apparently, two partially conflicting views exist about the origin of the nigral inhibitory GABA-ergic terminals, both supported by experimental evidence. It may be that the lesion of McGEER and co-workers, placed anterior to the globus pallidus, does not affect an important part of caudate-putamen as indicated in their figure 1 (McGEER et al., 1974). Yoshida suggests that monosynaptic inhibition in substantia nigra and globus pallidus originates from the same neuron in striatum and is inhibited by picrotoxine and bicuculline, but not by strychnine. Iontophoretically administered GABA or glycine also inhibits neuronal cell-firing in these regions. The inhibition by GABA is antagonized by bicuculline and/or picrotoxine, but not by strychnine (YOSHIDA, 1974). On the other hand, the action of glycine is antagonized by strychnine, but not by bicuculline or picrotoxine. Among the six types of nerve endings located on the dendrites and somata of nigral cells, less than 50% are of the type characterized by variable pleomorphic vesicles degenerating after stirato-nigral interruption (HASSLER, 1974). These characterized striato-nigral synapses are presumably GABA-ergic and probably affect the nigro-striatal dopaminergic projection as well as the presumably dopaminergic neurons that descend from the posterior nigra segment in the direction of the spinal cord influencing muscle tone (BAK et al., 1972; HASSLER, 1974). The GABA-ergic striato-nigral neurons may affect all efferent neurons of the substantia nigra. These studies indicate that a GABA-ergic dopaminergic interaction may occur at the level of the substantia nigra. At present it is not clear whether a direct contact exists between GABA-terminals and dopaminergic cell bodies or dendrites, or whether other intranigral pathways participate in the GABA-dopamine interaction in this region.

Caudate-putamen

The level of GABA in caudate-putamen is relatively low in most species, although moderate glutamate decarboxylase activity has been reported in this structure (LOWE et al., 1958; McGEER et al., 1971; MÜLLER & LANGEMANN, 1962). High levels of GABA-transaminase are found in human and monkey caudate-putamen (SALVADOR & ALBERS, 1959; SHERIDAN et al., 1967). Transsection of afferent pathways to caudate-putamen does not alter the glutamate decarboxylase activity in this structure (KIM et al., 1971; McGEER et al., 1974; HOCKMAN et al., 1971). This suggests that the GABA-ergic system in caudate-putamen is largely intrinsic. To date no evidence has been presented, other than indirect and speculative evidence, that these interneurons have specific functions in the extrapyramidal system. Electrophysiological experiments have not been reported for this area.

Globus pallidus

The globus pallidus in most primate species is composed of an external or lateral and an internal or medial segment, separated by a medullary lamina. In these species the pallidal segments and the outer putaminal part of striatum are classically referred to as the lentiform nucleus. Many striatofugal fibers converge through the pallidal segments, having collateral connections with cells of both pallidal segments (MEHLER & NAUTA, 1974).
Experiments of KANAZAWA & TOYOKURA (1974) and FONNUM et al. (1974) indicate that GABA and glutamate decarboxylase are unevenly distributed throughout the pallidal segments. The GABA-synthesising enzyme is highest in the ventrolateral segment (100%) and lower in the dorso-medial (69%), ventro-medial (35%) and dorso-lateral (7%) segment. A similar division is observed for the concentration of GABA in human brain (KANAZAWA & TOYOKURA, 1974). Lesions in the caudate nucleus result in small changes or no change at all in the ventro-lateral (–17%) or ventro-medial (–6%) area, while massive changes were observed in the dorso-medial (–75%) or dorso-lateral (–53%) segments (FONNUM et al., 1974). Glutamate decarboxylase activity is reduced in the lateral segment of the pars reticulata of substantia nigra by 55-80% only when the lesion is restricted to the caudal part of the caudate nucleus. Loss of glutamate decarboxylase activity is observed after complete destruction of nucleus caudates by 35-40% in globus phallidus medialis and by 40-60% for substantia nigra (KANAZAWA & TOYOKURA, 1974). Neuroanatomical evidence indicates that the major afferent input of the globus pallidus consists of collaterals of the massive system of striato-fugal fibers converging through it. Electrophysiological (YOSHIDA, 1974) and neurochemical (KANAZAWA & TOYOKURA, 1975) evidence suggests that this projection is GABA-ergic in nature. This is in accordance with the DALE principle indicating that collaterals release the same neurotransmitter as its neuron of origin, which releases GABA in substantia nigra. Picrotoxine and bicuculline depress the inhibition of cell-firing, observed in globus pallidus after stimulation of the caudate nucleus, while strychnine is ineffective (YOSHIDA, 1974). Iontophoretically applied GABA or glycine inhibits the firing of pallidal cells. The effect of GABA on cell-firing is only antagonized by picrotoxine or bicuculline. These experiments suggest that GABA is the neurotransmitter released by the collaterals of the striatofugal pathway projecting to the pallidum.

1.3.3. Comparison of the effects of GABA and glycine in other brain regions and the brain stem

In the brain stem, which has an intermediate position between the spinal cord

Table 1.3.2.
GABA in the brain stem and other brain regions

brain region	GABA concentration	GABA inhibition	antagonism	origin of GABA neurons	afferent lesions
(a) in the brain stem					
oculomotor nucleus	+	+	++ (p,b)	vestibular N.	+
trochlear nucleus	+	+	++ (p,b)	vestibular N.	+
superior colliculus	+	nt	nt	intrinsic	–
medullary reticular formation	nt	+?	+ (p)	ventral spinal cord	–
cuneate nucleus	nt	+	+ (p)	intrinsic	–
(b) in other brain regions					
olfactory bulb	nt	+	+ (b)	lateral olfactory tract	nt
retina	+	+	++ (p,b)	intrinsic	–
hippocampus and dentate gyrus	+	+	+ (b)	intrinsic	+

Abbreviations and symbols: GABA-concentration: + means that a high GABA concentration has been measured. GABA-inhibition: + means that GABA-mediated inhibition has been unquestionably detected; +? means a minor function of GABA as a transmitter. Antagonism: ++ (p,b) means that GABA-mediated inhibition is antagonized by picrotoxine (p) and bicuculline (b); + (p) or + (b) means that only picrotoxine or bicuculline has been tested or found effective. Afferent lesions: + means that GABA concentration decreases after afferent lesioning indicating that GABA-mediated inhibition is not only intrinsic. nt: not tested.

References:

Oculomotor nucleus: MIYATA et al., 1970; OBATA & HIGHSTEIN, 1970; HIGHSTEIN et al., 1971; OKADA et al., 1971; PRECHT & BAKER, 1972; FONNUM, 1972; OTSUKA & MIYATA, 1972; PRECHT et al., 1973.
Trochlear nucleus: similar as described for the oculomotor nucleus. Superior colliculus: ALBERS & BRADY, 1959; FAHN & COTE 1968; OKADA et al., 1971; OKADA & SHIMADA, 1976. Medullary reticular formation: TEBECIS & ISHIKAWA, 1973. Cuneate nucleus: GALINDO, 1969; DAVIDSON & SOUTHWICK, 1971; CURTIS & JOHNSTON, 1973. Olfactory bulb: NICOLL, 1971; McLENNAN, 1971; CURTIS & JOHNSTON, 1973; KELLY & BEART, 1975. Retina: KURIYAMA et al., 1968; GRAHAM et al., 1970; GRAHAM, 1972; GRAHAM & PONG, 1972a and 1972b. Hippocampus and dentate gyrus: CURTIS et al., 1970; KILMER & McLARDY, 1970; STORM-MATHISEN & FONNUM, 1971; STORM-MATHISEN, 1972; ITO, 1976; OKADA & SHIMADA, 1976.

and the cerebral hemispheres, glycine concentrations are progressively increasing, while those of GABA are progressively decreasing in a caudal direction. Concurrently, a high affinity uptake system for glycine appears in the pons and

medulla, suggesting that glycine is here, unlike in higher brain areas, more important as an inhibitory transmitter (JOHNSTON & IVERSEN, 1971; ARREGUI et al., 1972).
A summary of the available evidence for an inhibitory role of GABA in brain stem nuclei and some remaining higher brain regions is presented in Table 1.3.3. Among these brain areas and nuclei firm evidence for a GABA-mediated inhibition has been found in cerebellum and the trochlear and oculomotor nucleus. Not mentioned here, but of considerable importance is the sensitivity of the GABA-ergic system for physiological stimuli as has been reported for retina (GRAHAM et al., 1970; LAM, 1972; GRAHAM & PONG, 1972a and 1972b) and trochlear nucleus (WATANABA, 1971b; KURIYAMA & KIMURA, 1976).

1.3.4. GABA in autonomic or dorsal root ganglia

The levels of GABA in ganglia are very low and rule out a possible transmitter function of GABA in these structures. However, GABA depolarizes the ganglion while glycine is ineffective (DE GROAT, 1972; BOWERY & BROWN, 1972). From intracellular studies it appears that the membrane conductivity increases while a depolarization occurs, probably the result of an enhanced chloride-ion permeability (ADAMS & BROWN, 1973). These effects are antagonized by picrotoxine and bicuculline, but not by strychnine (DE GROAT, 1970a; DE GROAT et al., 1971; BOWERY & BROWN, 1972).

1.4. LOCALIZATION OF GABA-TRANSAMINASE AND SSA-DEHYDROGENASE

In this chapter the precise localization of GABA-transaminase and SSA-dehydrogenase in cellular structures, subcellular fractions or brain regions will be discussed, with the intention to demonstrate that the localization of these enzymes does not contradict a regulatory role of the GABA degrading enzymes in the concentration of GABA.

1.4.1. The activities of the enzymes of the GABA shunt

A large variation in activity has been reported for GABA-transaminase (SALGANICOFF & DE ROBERTIS, 1965; VAN KEMPEN et al., 1965; PITTS et al., 1965; COLLINS, 1972; BEART & JOHNSTON, 1972; WOOD & PEESKER, 1973). More consistent values for glutamate decarboxylase or SSA-dehydrogenase activity have been reported throughout the literature, although artefacts have interfered with the assays of both enzymes. For example, the presumed existence of a so-called GAD II may be due to the presence of an impurity in the radioactive glutamate sample used for the assay of this enzyme (see chapter 1.2.1). The high activity of SSA-dehydrogenase with increasing temperature resulting in an assay at 40°C (PITTS & QUICK, 1965) is possibly caused by incubation artefacts and the use of inappropriate blanks, as suggested by KAMMERAAT (1966). The most likely values for *in vitro* activities of the three GABA shunt enzymes discussed in this thesis are summarized in Table 1.4.1.

Table 1.4.1.

Activities of the GABA shunt enzymes measured *in vitro*

enzyme	activity at pH-optimum	activity at pH 7.4	authors
glutamate decarboxylase	25-35	24-30	a,b
GABA-transaminase	80-140	60-100	a,d,e,h
SSA-dehydrogenase	160-200	80-100	c,f,g

Activities are expressed as μmoles/g tissue (wet wt)/hr at 37°C under optimal conditions or at physiological pH, based on the following papers: a. ROBERTS & EIDELBERG, 1960; b. SALVADOR & ALBERS, 1959; c. EMBREE & ALBERS, 1964; d. WAKSMAN & ROBERTS, 1965; e. SUSZ et al., 1966; f. KAMMERAAT, 1966; g. PITTS et al., 1965; h. SCHOUSBOE et al., 1973.

1.4.2. The subcellular localization of the enzymes of the GABA shunt

The subcellular localization of the enzymes of the GABA shunt – glutamate decarboxylase, GABA-transaminase and SSA-dehydrogenase – have extensively been studied by SALGANICOFF & DE ROBERTIS (1963 and 1965). The findings of these authors are particularly valuable and the essential data are summarized below in Table 1.4.2. These studies demonstrate that glutamate decarboxylase is concentrated in nerve ending enriched fractions. GABA-transaminase and SSA-dehydrogenase are preferentially located in mitochondria, both of synaptosomal and non-synaptosomal origin. The ratio of enzyme activity recovered in fraction E (free mitochondria) as compared to fraction C plus D (mitochondria of synaptosomal origin) is 3.3 for GABA-transaminase, while for SSA-dehydrogenase a ratio of 1.9 has been observed. This indicates the presence of substantial amounts of the GABA-degrading enzymes in nerve ending preparations. Similar results in less extensive studies have been obtained by others for GABA-transaminase (VAN KEMPEN et al., 1965; WAKSMAN et al., 1968; FONNUM, 1968; KURIYAMA, 1976) and SSA-dehydrogenase (SIMS & DAVIS, 1973). The studies with SSA-dehydrogenase by SALGANICOFF & DE ROBERTIS lack an adequate SSA-dehydrogenase assay, but the experiments in the presence of triton X-100 – showing a similar distribution among fractions as GABA-transaminase – seem reliable. Similar, though less extensive studies of SIM & DAVIS (1973) and KAMMERAAT (1966) confirm the presence of SSA-

Table 1.4.2.

The localization of several enzymes in subcellular fractions

enzyme	MIT (P_2)	SYNAPTOSOMES (C + D)	FREE MITOCHONDRIA (E)	$\frac{E}{(C + D)}$	ACTIVITY
GAD	51	45	3	0.07	25.2
GABA-T	83	19	63	3.3	69.0
SSA-DH	77-*87*	21-*34*	38-*63*	1.8-1.9	142.2
SDH	85-*87*	49-*51*	31-*32*	–	–
GDH	82-*86*	23-*36*	42-*62*	1.8-*1.7*	–
ASP-AT	24-*47*	10-*26*	6-*14*	–	–
Alan-AT	42-*44*	22-*24*	15-*16*	–	–
protein	48	26	8	0.31	81.0

All italicised values indicate that the incubations have been performed after treatment of the tissue with triton X-100. The values for the first three columns indicate the percentages recovered in the two synaptosomal fractions as compared to the free mitochondrial fraction. The last column gives the values in μmoles/g tissue/hr for enzyme activities, while protein is given in mg/g tissue (from SALGANICOFF & DE ROBERTIS, 1965).

dehydrogenase in considerable amounts in fractions containing nerve endings. A comparison of the properties of GABA-transaminase in nerve ending containing fractions as compared to those in free mitochondria suggests the presence in the latter fraction of a GABA-transaminase that is not affected (or only slightly activated) by aspartate or glutamate in high concentration. The nerve ending derived fraction, however, contains GABA-transaminase activity that is markedly inhibited by aspartate and to a much lesser extent by glutamate. This suggests that both fractions contain different forms of GABA-transaminase (SALGANICOFF & DE ROBERTIS, 1965), According to WAKSMAN et al. (1968) it may be possible that the GABA-transaminase containing nerve endings are not GABA-ergic and do not synthesize GABA, but are inhibited by GABA-ergic neurons making presynaptic contacts. Therefore, these GABA-transaminase containing nerve endings may also be derived from excitatory terminals, which take up GABA from the extracellular fluid to terminate its neurotransmitter function and metabolize GABA. Evidence discussed further on will indicate that GABA-transaminase is specifically associated with GABA-ergic terminals as observed in lesion experiments in the substantia nigra (KATAOKA et al., 1974). However, this observation does not exclude the possibility suggested by WAKSMAN et al. (1968) that part of these GABA metabolizing enzymes are located in other than GABA-ergic terminals.
BUU & VAN GELDER (1974) have further substantiated the evidence for a localization of GABA-transaminase and SSA-dehydrogenase in nerve terminals. The nerve ending fraction and the mitochondrial fraction both obtained from a P_2-fraction contains transaminase activity with a different pH-optimum, different affinity for GABA and a differential sensitivity for two selective inhibitors of GABA-transaminase (BUU & VAN GELDER, 1974). This free mitochondria fraction contains two to three times as much activity – on a wet weight basis – as compared to the nerve ending fraction. The presence of the GABA degrading enzymes in nerve endings suggests that these two enzymes may contribute to the control of the concentration of GABA in these terminals. Possibly five GABA-transaminase isozymes may be present with different mobility upon electrophoresis (WAKSMAN & BLOCH, 1968). One of the forms, isolated by BUU & VAN GELDER (1974) is probably identical to one of the fractions mentioned above.

1.4.3. The regional distribution of the enzymes of the GABA shunt

The regional distribution of the enzymes of the GABA shunt has been recently reviewed (FAHN, 1976). Most aspects of the regional distribution have already been discussed in the previous chapter. It was concluded that no association exists between the level of GABA and the activities of GABA-transaminase or

SSA-dehydrogenase (ROBERTS & EIDELBERG, 1960; TAPIA, 1975). In addition, both enzymes involved in the degradation of GABA seem to be similarly distributed. However, close examination of the detailed experiments of PITTS and co-workers (SHERIDAN et al., 1967; MILLER & PITTS, 1967) on human brain GABA-transaminase and SSA-dehydrogenase reveals significant differences in the ratio of GABA-transaminase and SSA-dehydrogenase activities. For example, although in general a ratio of 0.6 has been observed in most brain regions and total brain homogenates, a ratio of 1.15 has been observed in the globus pallidus. The relevance of this observation is not clear, since no data are available about other differences in localizations of GABA-transaminase and SSA-dehydrogenase activity.
The absence of any correlation between the levels of GABA degrading enzymes and the concentration of GABA is hardly surprising. Recent findings of TAPPAZ et al. (1977) indicate that the correlation between GABA concentration and glutamate decarboxylase may result from post-mortem synthesis of GABA. It can therefore not be excluded that this post-mortem synthesis of GABA covers an underlying correlation between GABA concentration and GABA degrading enzymes.

1.4.4. The localization of GABA-transaminase and SSA-dehydrogenase in cellular structures

The functional importance of GABA-transaminase has been studied by measuring enzyme levels in well-defined structures associated with different functions, using histochemical as well as sensitive biochemical methods. Because SSA-dehydrogenase is also needed for most of the histochemical reactions, these histochemical studies do not measure GABA-transaminase exclusively. Therefore, GABA degradation via both GABA-transaminase and SSA-dehydrogenase is studied with these methods. Immunohistochemical methods have been used mostly to trace the localization of glutamate decarboxylase, while some preliminary reports have appeared concerning the localization of GABA-transaminase. This type of study permits the localization of GABA-transaminase – or a particular form of GABA-transaminase – without the necessity of coupling it with SSA-dehydrogenase. Some histochemical studies have also been reported for SSA-dehydrogenase.
GABA degradation is not uniformly distributed throughout the different functional layers of the cerebellum, cortex and hippocampus (SALVADOR & ALBERS, 1959). Similar differential localizations are observed for GABA degradation in many brain nuclei, while a distinction has also been made between nerve cell bodies and glial cells (ROBINSON & WELLS, 1973). The rate of GABA degradation is measured with histofluorescence techniques after incuba-

tion of brain slices in a GABA containing medium. A high rate of GABA degradation is observed in the caudate-putamen, nucleus accumbens septi and in layers of cerebellum. Low rates of GABA metabolism are found in most diencephalic and mesencephalic muclei. The rate of GABA metabolism in glial structures is low and seldom exceeds GABA metabolism in nerve cells.

In the cerebellar layers a differential localization has been found for GABA-transaminase using histofluorescense or a micro-assay for GABA-transaminase (VAN GELDER, 1965; PITTS et al., 1965; KURIYAMA et al., 1966; ROBERTS & KURIYAMA, 1968). GABA-transaminase is concentrated in the molecular layer while lower levels are observed in the granular layer. GABA degradation is also high in Purkinje cell cytoplasma, golgi cell bodies and mossy fibre endings. The presumed excitatory granule cells show either no reaction, or a low rate of GABA metabolism (VAN GELDER, 1965; KURIYAMA et al., 1966; ROBERTS & KURIYAMA, 1968). Glial cells do not react intensively in the histochemical GABA-transaminase reaction (HYDE & ROBINSON, 1974a and 1974b). Somehwat different results were obtained by the latter group in cerebellum. Intense metabolism is observed in Purkinje cells and their dendrites, the synaptic glomeruli and in the cytoplasm of the granule cell bodies in the granular layer. No reaction is observed in stellate, basket and golgi interneurons (HYDE & ROBINSON, 1974a). This study indicates that only a minority of cells are capable of metabolizing GABA in cerebellum.

Separate measurements of SSA-dehydrogenase show that its distribution throughout layers of cerebellar cortex is similar to that of GABA-transaminase (PITTS & QUICK, 1965). This localization is confirmed in an elegant study demonstrating histochemically the association of SSA-dehydrogenase with the dendritic tree of Purkinje cells (SIMS et al., 1971). SSA-dehydrogenase is especially localized in areas with dense projections, where its concentration is higher in nerve endings than in cell bodies. A high SSA-dehydrogenase activity partially correlates with the activity of succinic dehydrogenase, suggesting that the dichotomous functions of GABA in the brain (transmitter or intermediary metabolite) are interrelated. In addition, it is suggested that a high activity in cell bodies may indicate a GABA receptor cell, a GABA-ergic cell or a high rate of enzyme synthesis. According to these authors the method of VAN GELDER (1965), which is used by many authors mentioned above, may give wrong results because the formazon-producing diaphorase reaction may be rate-limiting (SIMS et al., 1971).

Several methods have been described to isolate different brain structures in separate fractions, among which the method of HAMBERGER and co-workers proved to be particularly valuable (HAMBERGER & SELLSTRÖM, 1975). They have found that glutamate decarboxylase activity in partially purified glial cells or neuronal material is about 10% of the activity in synaptosomes. For GABA-transaminase a completely different picture is obtained. The highest GABA-

transaminase activity is present in the glial preparation, the synaptosomal preparation is intermediate, while the activity in the neuronal fraction is 50% of that observed in the glial cell containing fraction (HAMBERGER & SELLSTRÖM, 1975). Though the synaptosomal fraction may be contaminated with up to 40% of gliosomes (so-called pinch-off structures of glial origin), which may possess GABA-transaminase activity, this contamination is probably too small to account for the relatively high activity of GABA-transaminase in synaptosomal fractions (SELLSTRÖM et al., 1975). Moreover, gliosomes do not contain very much mitochondria, where GABA-transaminase is localized (COTMAN et al., 1971). Experiments of KAMMERAAT (1966) indicate that SSA-dehydrogenase is present in partially purified glial cells, but the results are not very detailed in this respect.

1.4.5. Possible function of GABA-transaminase and SSA-dehydrogenase in the regulation of presynaptic GABA levels

As compared to other brain nuclei the substantia nigra is very rich in glutamate decarboxylase activity, but not in GABA-transaminase activity (KATAOKA et al., 1974; SALVADOR & ALBERS, 1959). Hemisection at the level of the subthalamic nucleus results in a 70% decrease of glutamate decarboxylase activity and a 25% decrease of GABA-transaminase activity (KATAOKA et al., 1974). Calculations show that between 25 and 36% of the GABA-transaminase is probably of synaptic origin. So far, no such data have been produced for SSA-dehydrogenase.

Differential centrifugation studies consistently suggest that part of the GABA-transaminase and SSA-dehydrogenase is located in nerve ending enriched fractions. Although glial contamination cannot be ruled out completely as a possible cause for these findings (COTMAN et al., 1971; HENN et al., 1976), contamination from other mitochondria containing fractions may also account for this finding. However, the lesion experiments provide convincing evidence for the presence of relatively large amounts of GABA-transaminase in nerve endings. These studies have been performed in monkeys, where GABA-transaminase is about 96 μmoles/g tissue/hr and glutamate decarboxylase activity is about 35 μmoles/g tissue/hr. Supposing that glutamate decarboxylase is about 60% active in the absence of exogenously added PLP-cofactor, this suggests the presence of an *in vivo* capacity of synthesising 21 μmoles/g.tissue/hr. Comparable levels for synaptosomal GABA-transaminase can be calculated using a 25% recovery of the activity of this enzyme in synaptosomal fractions.

These experiments suggest a role of GABA metabolism in the presynaptic regulation of the concentration of GABA. Such a role has so far been neglected in the literature. No data have been reported on the effect of lesions on SSA-dehydrog-

enase using methods as described above for GABA-transaminase. Such lesion studies using the well-defined striato-nigral bundle may prove very useful in establishing the functional importance of SSA-dehydrogenase in the regulation of pre-synaptic GABA.

1.5. ACUTE BEHAVIOURAL EFFECTS OF OPIATES

The principle pharmacological actions of narcotic agonists include effects on the central nervous system and gastrointestinal effects. In this chapter the acute effects of morphine on the central nervous system will be briefly discussed.

1.5.1. Depressant and stimulant actions of narcotic agonists

Morphine and related narcotic agonists produce depressant and/or stimulant effects in many species in a time and dose dependent manner (DOMINO et al., 1976a). In the rat morphine has initial depressant and subsequently stimulant properties (DOMINO et al., 1976a and 1976b). Measurement of locomotor activity with 1, 3.2, 10 and 32 mg/kg morphine results in an initial depressant and subsequently activatory phase, with an increase in duration of the first depressant phase with dosage (DOMINO et al., 1976a and 1976b). For example, with 20 mg/kg morphine an initial depressant or cataleptoid state is observed, followed by a subsequent stimulation of motor activity. The duration of the initial cataleptoid state is dose dependent. These biphasic effects of opiates are observed on locomotor activity in the rat (references, see DOMINO et al., 1976a) and comparable effects are observed in the cat (COOLS et al., 1974), on self-stimulation in the rat (HOLTZMAN & JEWETT, 1972; LORENS & MITCHELL, 1973), on EEG phenomena (GUNNE, 1960; COLASANTI & KHAZAN, 1973; MORETON et al., 1975) and body temperature (CHAHOVITCH & VICHNJITCH, 1928; HERMANN, 1942). It is important to account for differential effects of morphine with respect to: animal species, dose and route of administration, time after administration and the behavioural state of the animal. AYHAN & RANDRUP (1973a and 1973b) have observed that the behavioural effects of morphine are less when morphine is injected in the morning. When given at 8.30 a.m. morphine (2 mg/kg) produces a significant depression of locomotor activity and rearing during the first 30 min after injection, which is followed by a period of stimulation (AYHAN, 1974). However, when given at any later time until 23.30 injection of morphine produces no depression of activity during the first 30 min observation period. At any time increased activity is observed during subsequent observation periods. Several investigations have demonstrated that tolerance develops to the depressant effects of morphine, viz. analgesia, hypothermia and decrease in locomotor activity (SEEVERS & DENEAU, 1963; MARTIN et al., 1963; HOSOYA et al., 1963; OKA et al., 1972a and 1972b). The observation that naloxone injected 30 or 60 min after morphine prevents both the depressant and stimulant effects of morphine on locomotor activity suggests that both effects are mediated by the same receptor (OKA & HOSOYA, 1976). The involvement of different neuronal mechanisms in

the stimulant and depressant effects of morphine suggests that these effects of morphine are mediated via different mechanisms probably located in different brain regions (OKA & HOSOYA, 1976).
The integrity of specific brain nuclei is important for the acute effects of morphine (BROEKKAMP et al., 1976; COSTALL et al., 1976; PYCOCK & HORTON, 1976; PERT & SIVIT, 1977). Injections of morphine into the posterior hypothalamus produce an increase in the rate of self-stimulation, whereas injections into the locus coeruleus and periaquaductal grey matter are inhibitory. Injections into the ventricular system or into structures between the sites for the stimulant and depressant effects of morphine produce biphasic responses: inhibition followed by excitation (BROEKKAMP et al., 1976). Similar biphasic effects are observed after injection of morphine into the nucleus accumbens mediating depression, viz. catatonia, and hyperactivity, viz. stereotyped biting (COSTALL et al., 1975b). Injection of morphine or D-ala-enkephalin-amide (an enzyme-resistant enkephalin with morphine-like properties, see further) produce a gradual increase of locomotor activity, whereas 10 mg/kg naloxone produces hypomotility. Apomorphine, a dopamine agonist, produces an immediate increase in activity when injected into this area, which is not antagonizes by haloperidol (PERT & SIVIT, 1977). This dopaminergic system mediating the increase of locomotor activity after injection of dopamine into the nucleus accumbens is probably controlled by some GABA-ergic system, because the GABA-transaminase inhibitor ethanolamine-O-sulphate inhibits the response to dopamine (PYCOCK & HORTON, 1976). Whether GABA is involved in the effects of morphine mediated via this brain region has not been studied.
In conclusion, the depressant and stimulant effects of acutely administered morphine can be clearly separated with respect to development of tolerance to the observed effect, the localization in brain regions and probably also the involvement of specific neuro-transmitter systems.

1.5.2. The analgesic action of narcotic agonists

Apart from the behavioural effects already discussed in chapter 1.5.1. two properties of narcotic agonists, associated with acute administration, are most important: its analgesic action and its euphoric action. Both actions of narcotic agonists have an important scientific and social bearing.
The analgesic action of morphine (after administration of 3 to 10 mg/kg) or related substances can be measured in laboratory animals using tests such as the tail flick test, the hot plate test, the tail pressure test etc. (review, see KNOLL, 1975). A recently published method may allow the evaluation of the analgesic action of morphine on different levels of the CNS (PAALZOW & PAALZOW, 1973; PAALZOW et al., 1974; DAHLSTRÖM et al., 1975). In untreated animals

three responses appear: firstly, a motor response (spinal reflex); secondly, a vocalization response (mediated by certain structures in the medulla oblongata); and finally, a vocalization after-discharge (vocalization of the rat after withdrawal of the painful stimulus). This vocalization after-discharge in the rat may involve the emotional component of pain according to these authors (DAHLSTRÖHM et al., 1975). It is, however, beyond the scope of this introduction to go into further detail here. Many aspects will be further discussed in the following chapters, where analgesia is often used as end point to characterize acute and chronic effects of opioids.

1.5.3. Schedule-controlled operant behaviour and narcotic agonists

In a recent review three general areas of behavioural methodology are discussed: schedule-controlled operant behaviour, sensitive to the actions of narcotic agonists; opiate agonists as discriminative stimuli for operant behaviour; and narcotic agonists as reinforcing stimuli of operant behaviour (SCHUSTER, 1976). Such type of studies have been used successfully to study the interactions between narcotic agonists and partial agonist-antagonists, demonstrating the usefulness of these studies in obtaining stable baseline responses. Narcotic agonists have the capacity to serve as discriminative stimuli for maze performances and lever-pressing tasks (BELLEVILLE, 1964; HILL et al., 1971; ROSECRANS et al., 1973; OVERTON, 1974; HIRSCHHORN & ROSECRANS, 1974; KUHN et al., 1976). The ability of morphine to function as a discriminative stimulus in the rat may be analogous to its ability to produce subjective effects in man (SCHUSTER, 1976. Drugs commonly abused by man serve as reinforcing stimuli in laboratory animals producing high rates of opiate self-administration (SCHUSTER & THOMPSON, 1969; SCHUSTER & JOHANSON, 1974), while drugs with subjectively dysphoric properties in man act as negative reinforcers in laboratory animals (GOLDBERG et al., 1971; HOFFMEISTER & WUTTKE, 1974). Therefore, these properties of abused drugs can be studied effectively using their reinforcing properties in laboratory animal experiments.

1.6. CHRONIC BEHAVIOURAL EFFECTS OF OPIATES

Chronic treatment with opiates results in the development of tolerance to, and physical dependence on, these opiates. Tolerance, because increasing amounts of the opiate are required to obtain the same magnitude of response: for example, the suppression of a painful stimulus or obtaining a euphoric effect; physical dependence, because the opiate is needed in increasing amounts to allow a more or less normal functioning of the organism. Terminating the chronic treatment with opiate may cause an abstinence syndrome, a predictable and highly characteristic pattern of behaviour. The unpleasant character of this abstinence syndrome is an important factor in the development of addiction, since the continuation of opiate administration results in an immediate suppression of this abstinence syndrome. Instead, an appropriate dose of opiate will cause euphoria within seconds after administration. In this chapter physical dependence after chronic treatment with opiate and the relationship with tolerance development will be discussed.

1.6.1. The measurement of physical dependence

MARTIN (1967) defined physical dependence as follows: "Precipitated or acute abstinence refers to an abstinence syndrome produced by a morphine antagonist such as nalorphine in either acutely or chronically physically dependent animals. Further, the precipitated abstinence syndrome refers to any group of signs which cannot be attributed to a combination of the independent actions of the narcotic and the narcotic antagonist. By acute dependence is meant a state in which abstinence can be demonstrated or precipitated following either a single dose or a short-term infusion of morphine. In contradiction, chronic physical dependence designates a state in which an abstinence syndrome can be precipitated or becomes manifest when the drug is withdrawn following a prolonged course of administration of morphine or similar agents. Withdrawal or chronic abstinence refers to an abstinence syndrome that becomes manifest when morphine-like agents are withdrawn from physically dependent animals."
Physical dependence indicates that normal functioning of the organism is only possible in the presence of certain amounts of opiate. Assessment of the degree of dependence can thus, by definition, only be performed by withdrawal of the opiate, either by stopping the chronic treatment with opiate, or by administration of an opiate antagonist. The first method is called abrupt withdrawal (see above) and the abstinence syndrome develops slowly reaching maximum intensity some hours after discontinuing opiate administration. The second method is called precipitated withdrawal, and can be achieved by administration of a partial agonist-antagonist, or a pure antagonist of opiates. In this case the abstin-

ence syndrome develops more quickly, is more intense and shorter of duration than the syndrome observed after abrupt withdrawal.

1.6.2. The production of highly tolerant and dependent subjects

Physical dependence on, and tolerance to, narcotics has been produced in animals by a variety of techniques, including intravenous self-administration (GOLDBERG et al., 1971), oral self-administration (STOLERMAN & KUMAR, 1970), systemic injection using a multiple injection schema (MARTIN et al., 1963) and by way of intraventricular injection (EIDELBERG & BARSTOW, 1971). WAY and co-workers have introduced the pellet method to make rats or mice dependent on morphine by modifying a method originally proposed by Huidobro & Maggiole (HUIDOBRO & MAGGIOLO, 1961; WAY et al., 1969; GIBSON & TINGSTAD, 1970; CICERO & MEYER, 1973; WEI & WAY, 1975). This pellet, containing 75 mg of morphine-base, releases morphine over a large period of time. Consequently, a high degree of tolerance and physical dependence develops within three days after subcutaneous implantation (WAY et al., 1969; WEI & WAY, 1975). Physical dependence is demonstrated by the removal of the pellet (abrupt withdrawal) or by injecting small amounts of naloxone (precipitated withdrawal). COLLIER et al. (1972) used the injection of 150 mg/kg morphine in a sustained release preparation to obtain highly dependent animals in a relatively short period of time. WEI (1976) has recently introduced a method for the continuous administration of opiate into the intraventricular system of the rat allowing the use of small amounts of opiates to induce dependence.

1.6.3. Characterization and measurement in practice of physical dependence

Precipitated abstinence in mice is characterized by defecation, urination, increased locomotor activity, tremors, sometimes convulsions, and most characteristically by stereotyped jumping (WAY & LOH, 1976). The morphine abstinence syndrome in the rat is different. It is most reliably characterized by weight loss, hypothermia and the incidence of head and body shakes, the so-called wet dog shakes. To date extensive scoring methods are used to characterize the morphine abstinence syndrome. BLÄSIG et al. (1973) distinguish two classes of abstinence signs, viz. dominant signs like jumping, flying or teeth chatter developing mostly after about 10 to 15 min during precipitated abstinence, and recessive signs, like writhing and shaking. The latter signs develop early after precipitation of abstinence behaviour, but are replaced by the dominant signs later on. A distinction has been made between autonomic and central signs (FREDERICKSON

& SMITS, 1973; JHAMANDAS, 1973; FREDERICKSON, 1975). Lacrimation, diarrhoea, salivation etc. are classified as autonomic signs, while shaking, yawning, digging, hunch back posture, ptosis etc. are described as central signs. The degree of dependence in the mouse has been quantified by measuring the ED_{50} of naloxone required to precipitate the response, viz. escape jumping of mice from a platform (WAY et al., 1969). WEI (1973) has introduced a ranking system for qualifying precipitated withdrawal in rats, giving points for the incidence of certain abstinence signs: 1 point for abnormal posturing, ear blanching and diarrhoea (any 2 out of 3 abstinence signs), 2 points for teeth chattering or swallowing or salivation, and 3 points for 3 or more wet shakes or two or more escape attempts. Comparable methods have been introduced by others based upon scoring systems accounting for the many abstinence signs observed during precipitated withdrawal in morphine dependent rats (WATANABA, 1971a; COLLIER et al., 1972; BLÄSIG et al., 1973; FREDERICKSON & SMITS, 1973). A reliable estimate of the degree of physical dependence in rats is the ED_{50} of naloxone required to precipitate the abstinence syndrome quantified as described above (WEI et al., 1973).

Morphine abstinence is not characterized by unique behavioural items. Many other treatments may result in the development of certain signs and many of them, including digging, shaking or yawning are occasionally observed in normal animals. However, both the combination of all these signs and the much higher incidence of these signs characterize the morphine abstinence syndrome. The pattern is also specific for certain species. In the cat intense miaouing is often observed, while in the monkey irritation and yawning are characteristic abstinence signs. In man, morphine abstinence is characterized by yawning, sweating, irritation and fear (MARTIN, 1967).

It is possible to measure dependence after a single dose of morphine. A comparison of this single-dose dependence with dependence induced by the pellet method in mice, indicates that both syndromes precipitated by antagonist administration, can be differentially affected by various pharmacological treatments (TAKEMORI et al., 1976). Though escape jumping has been considered as a reliable parameter for measuring the degree of dependence in mice and rats, it must be appreciated that jumping cannot reliably and in a dose-dependent manner be induced in rather low-dependent rats and mice (BLÄSIG, 1976). Moreover, the value of scoring systems using several withdrawal signs is restricted. Withdrawal signs that characterize a low degree of dependence can be partly replaced by other signs with an increasing degree of dependence (BLÄSIG et al., 1973; BLÄSIG, 1976). This is particularly important when the effect of drugs on the development of tolerance and physical dependence is studied and different degrees of dependence must be compared. It is also important to stress the differences between abrupt and precipitated withdrawal, which have a different intensity and time course (BLÄSIG, 1976).

1.6.4. The development of tolerance and physical dependence

The development of tolerance and physical dependence in mice associated with the chronic administration of morphine develop concurrently. This is indicated by the parallelity of the changes in ED_{50} values for naloxone precipitating withdrawal signs and the ED_{50} values for morphine giving tolerance (WAY et al., 1969). A similar parallel development of tolerance and physical dependence is also observed in the rat (CICERO & MEYER, 1973), using the number of wet shakes of the rat as an indicator of the degree of dependence. Bilateral destruction of the ventral noradrenergic bundle, which selectively depletes noradrenaline from the hypothalamus and thalamus, reduces tolerance, withdrawal symptoms, abstinence symptoms and opioid consumption concurrently in addicted rats (LEWIS et al., 1976). By co-administration of morphine and the partial agonist-antagonist nalorphine a complete blockade of tolerance (15 mg/kg) has been obtained (COCHIN & MUSHLIN, 1976). Administration of naloxone results in precipitated abstinence and weight loss, indicating that the animals are still physically dependent. This suggests that the blockade of tolerance development has been separated from the blockade of physical dependence in these experiments.
The value of such conclusions, however, depends completely on the reliability of the methods used to estimate tolerance and physical dependence. It is important, especially when one sign is selected for the characterization of dependence using precipitated abstinence (WAY et al., 1969; CICERO & MEYER, 1973), to note that a sign like jumping cannot be induced dose-dependently in low-dependent rats or mice, even with high doses of naloxone (BHARGAVA, 1977b; BLÄSIG et al., 1973; BLÄSIG, 1976). These and many other factors (BLÄSIG, 1976) have to be critically considered when taking the expression of withdrawal as the strength of the underlying dependence.

1.6.5. The localization of sensitive sites for the effects of morphine and naloxone in the brain

Many authors have observed the pharmacological effects of morphine after intraventricular injection (antinociception: TSOU & JANG, 1964; HERZ et al., 1970; JAQUET & LAJHTA, 1973; YAKSH & PERT, 1974, inhibition of respiration: FLOREZ et al., 1968; hyperglycemia: BORISON, 1962; FELDBERG & GUPTA, 1974, hypothermia: LOTTI et al., 1965; neuro-endocrine effects: LOMAX et al., 1970; catalepsy: BANERJEE et al., 1968). The precise localization of the site of action of morphine in the brain has been the subject of many studies. WEI and co-workers have ranked the sensitivity of the brain areas for the inhibition by morphine of shaking in pentobarbital anaesthetised rats in ice-

water, which is considered to be a painful stimulus (WEI et al., 1975a). Morphine is highly active in the area of the peri-aquaductal grey, the medial preoptic area and the locus coeruleus. It is less active in the nucleus accumbens, the fasciculus retroflexus, the medial thalamus and the septal area while a very low sensitivity for morphine is observed in various hypothalmic nuclei, basal ganglia, reticular formation, substantia nigra and the reticular nucleus of the thalamus (WEI et al., 1975a). In accordance with these findings, experiments in morphine dependent rats have shown that abstinence signs can be precipitated easily and reproducibly by minute amounts of naloxone in the areas around the medial thalamus and the diencephalic-mesencephalic juncture. Other areas, including the hypothalamus and lateral hypothalamus are not sensitive to naloxone and no severe abstinence can be precipitated there (WEI et al., 1972; WAY et al., 1974). More recently, data have been published indicating that two sites in the brain of dependent rats are very sensitive to naloxone, viz. the medial hypothalamus and the space around the periaquaductal fourth ventricle (WEI et al., 1975b). Intermediate areas are not sensitive to naloxone, suggesting that two separate sites in the brain may mediate naloxone precipitated shaking in morphine dependent rats. Wet shaking in ice-water is inhibited by morphine most effectively in the anterior, as opposed to the medial, diencephalic area, while the opposite is observed for the inhibition by morphine of naloxone elicited shaking in dependent rats (WEI et al., 1975a and 1975b). Analgesia after morphine administration is most reliably antagonized by naloxone injections in the medial thalamus and the medial midbrain (COLLINS et al., 1974). These experiments have been interpreted as an indication that morphine and naloxone have different sites of action. It must be noted that only escape attempts or wet shakes are systematically observed, though administration of naloxone to morphine dependent rats into the medial thalamic area produces most of the abstinence signs (WEI et al., 1972). Thus the medial thalamic area is the site of the brain most sensitive to naloxone, though a high sensitivity is also observed in areas surrounding the diencephalic-mesencephalic junctures.

Many studies point to the periaquaductal grey as one of the sites being most sensitive to the effects of morphine (see WEI et al., 1975b). Experiments of LASCHKA et al. (1976) indicate that injection of a partial agonist-antagonist into the fourth ventricle or its surroundings of morphine dependent rats produces severe abstinence behaviour. The effect in the medial thalamus is only weak to moderate, due to the absence of strong signs such as jumping or lacrimation. Subsequent control studies indicate that leakage into the ventricular system correlates with the incidence of abstinence signs after injection of labelled antagonist into the medial thalamic nucleus. It is concluded from these latter studies that the site of morphines nociceptive action in the brain of rats coincides with the site of highest sensitivity to morphine antagonists in morphine dependent rats (LASCHKA et al., 1976). In the homeostatic theory of dependence and

withdrawal (HIMMELSBACH, 1943) the hypothalamus is an important link. Initial studies have indicated that lesions of the ventromedial hypothalamus greatly reduce the withdrawal reaction (KERR & POZUELO, 1971), but recent studies using similar lesions fail to reproduce this finding (LINSEMAN, 1976). The latter study is in line with the initial observations of WEI et al. (1972) and LASCHKA et al. (1976) demonstrating that the hypothalamus is not sensitive to morphine antagonists. On the other hand, it has been shown that medial thalamic lesioning selectively affects tolerance to morphine without affecting precipitated withdrawal (TEITELBAUM et al., 1974). This is in direct conflict with the observation of FREDERICKSON et al. (1975) that electrical stimulation of the medial thalamic region of naive rats produces "a series of responses reminiscent of the morphine WD (withdrawal, this author) syndrome".

In conclusion, these experiments demonstrate that tolerance and physical dependence can be most reliably established in the periaquaductal grey and the area surrounding the fourth ventricle, suggesting that both phenomena originate from the same area. Though the medial thalamic area is not very sensitive to morphine in producing analgesia and probably also not very sensitive for morphine antagonists precipitating withdrawal signs, several experiments suggest that this region may be involved in the expression of at least some of the abstinence signs, including shaking behaviour (FREDERICKSON et al., 1975) and morphine tolerance (TEITELBAUM et al., 1974).

The large number of conflicting data observed in the above mentioned studies on the localization of the site of action of morphine and naloxone in specific brain areas may be explained by the conclusion of LASCHKA et al. (1976) that morphine may have many sites of actions in the brain and that morphine abstinence signs may originate from different brain areas. Therefore, more consistent results may be obtained by comparing individual signs as suggested by BLÄSIG et al. (1973) for a great number of behavioural items, instead of the method of Way and co-workers of measuring only jumping in mice, or shaking in rats.

No withdrawal reaction has been obtained by injecting levallorphan unilaterally into the nucleus caudatus putamen or globus pallidus (LASCHKA et al., 1976), areas which are very rich in stereospecific opiate binding sites (KUHAR et al., 1973; PERT et al., 1975). Therefore, it is not clear whether all these opiate binding sites are important for the action of morphine in the CNS

1.7. THE OPIATE RECEPTOR

1.7.1. Properties of the opiate receptor

The binding of opiates to specific receptors in the brain and spinal cord is an important step involved in the action of narcotic analgesics. Opiate binding receptors have been demonstrated in vertebrates but they are not present in invertebrates (SNYDER & SIMANTOV, 1977). The opiate receptor has some properties in common with other neurotransmitter receptors, such as differential modes of binding of agonists and antagonists and selective effects of ions on binding (PERT & SNYDER, 1973a; TERENIUS, 1973; SIMON et al., 1973; SNYDER, 1975). The latter effect is supposed to be related to the ionic mechanisms underlying synaptic transmission (SNYDER & SIMANTOV, 1977). Specific opiate receptor binding, characterized as the difference between the radioactive opiate binding without or with an excess of non-radioactive opiate, correlates closely with the pharmacological activity of opiates (PERT & SNYDER, 1973b; TERENIUS, 1973; SIMON et al., 1973).

The presence of sodium ions during incubation with the radioactive opiate causes an increase of 160% in the binding of opiate antagonists such as naloxone, while the binding of agonists sharply decreases to about 20 to 30% of controls (PERT & SNYDER, 1974; SIMON et al., 1973). Lithium ions are far less active in this respect. Pure agonists such as morphine become 12 to 60 times weaker bound when incubated in the presence of sodium ions, while the partial agonist-antagonist pentazocine is about 2 to 3 times less bound when incubated in the presence of sodium ions (SNYDER & SIMANTOV, 1977). A method which is based upon the differential effect of sodium ions on agonist or antagonist binding has been proposed to estimate the relative agonist-antagonist properties of opiates *in vitro* using this discriminating property of sodium (PERT & SNYDER, 1974). The mixed agonist-antagonist pentazocine, a presumed non-addictive analgesic, is 3 to 7 times weaker bound when incubated in the presence rather than the absence of sodium. Therefore it is suggested that the sodium effect on binding of partial agonist-antagonists may have practical relevance for the search for safer, non-addicting analgesics (SNYDER & SIMANTOV, 1977). Binding values of 100 nM are found for morphine in the presence of 100 mM NaCl, giving 50% displacement of stereospecific ^{3}H-naloxone binding (PERT & SNYDER, 1974). This value is similar to the value of morphine required to reduce the analgesic threshold by 50% in the brains of rats (ADLER, 1963). This suggests that the binding of opiate agonists in the presence of sodium ions correlates better with their pharmacological activity than the binding in the absence of sodium ions. In addition, this correlation between the affinity of opiate agonists for the opiate receptor in the presence of NaCl with the levels of opiate agonists required for an analgesic action, suggests that the opiate receptor is

located on the outside of the membrane of the nerve terminals, where the sodium ion concentration is high.

The results mentioned above are consistent with a two-state model for the opiate receptor with the antagonists displaying selective affinity for the sodium state of the receptor, while the agonists prefer a "no sodium" state (PERT & SNYDER, 1974; SIMON et al., 1975). In this model sodium ions are supposed to affect the conformation of the opiate receptor causing an allosteric change of the receptor molecule form an "agonist prefering" towards an "antagonist prefering" conformation. Independent evidence for such a model is provided by inactivation experiments using sulfhydryl reagents, which behave differently in the presence, as compared to the absence of 100 mM NaCl (SIMON & GROTH, 1975; SIMON et al., 1975). It is concluded that the receptor may exist in two forms in the absence of sodium (dimer and monomer, or any other conformationally different form). The dimer is thought to be readily convertible to the sodium-dependent form, while the monomer is converted very slowly. The residual sodium-free conformation persisting in 100 mM NaCl may then be the monomer. In agreement with this model allosteric changes observed as the Scatchard plots of saturation data provide evidence for cooperativity (SIMON et al., 1975). A somewhat different position is taken by Snyder and co-workers who suggest that residual binding sites after treatment with sulfhydryl-reagents, are "frozen" in a conformation prefering the antagonist, viz. the sodium-dependent state (PASTERNAK & SNYDER, 1975). Apart from these differences, results comparable with those obtained by Simon and co-workers for the effect of sulfhydryl-reagents have been reported by Snyder and co-workers (WILSON et al., 1975; PASTERNAK & SNYDER, 1975; PASTERNAK et al., 1975a). Experiments with bivalent cations indicate that the opiate receptor function may be partially regulated by endogenously present bivalent cations (PASTERNAK et al., 1975b). These effects of bivalent cations are maximal in the presence of NaCl.

The concentration of extracellular sodium ions is high *in vivo*. This, together with the characteristics of opiate agonist or antagonist binding in the presence of relatively high concentrations of sodium ion (50 to 100 mM) suggests that under these conditions the state of the opiate receptor reflects most reliably the *in vivo* effects of acutely administered opiate agonists or antagonists (SNYDER & PASTERNAK, 1977). In this respect it is of interest to note that it has been postulated that a marked sodium deficiency may occur during depolarization near the presynaptic membrane of catecholamine containing nerve terminals (BRUINVELS, 1975). Repititive stimulation of axon-terminals may create a state of sodium deficiency which may either considerably promote the binding of an opiate agonist or cause a natural ligand for the opiate receptor to be released from presumed peptidergic neurons (see also further) *in vivo*. This may result in presynaptic inhibition of the repititive firing neuron. The efficacy of inhibition

will depend on the extent of sodium deficiency and thus on the firing rate of this particular neuron. At present, such a model suggesting a function of opioids in the regulation of catecholaminergic neurons is purely speculative.
Within 5 min, the administration of 50 mg/kg morphine sulphate *in vivo* increases the stereospecific binding of ^{3}H-dihydromorphine to the opiate receptor to the opiate receptor by about 70%, while after 60 min a 30% increase can be detected. Similar results are obtained between 2 and 108 hrs after pellet implantation (PERT & SNYDER, 1976). This time course does not correlate with the development of tolerance and physical dependence. The enhancement of receptor binding by antagonists is 10 to 1000 times as large as the enhancement by their corresponding agonists. It is concluded that the number of free binding sites for opiates is increased by this treatment *in vivo*, probably by unmasking receptor sites normally occupied by opioid peptides (PERT & SNYDER, 1976).
At present no clear cut changes in number of binding sites or properties of the opiate receptor have been noted with the chronic treatment with opiates, even when specific brain regions which have a high proportion of opiate receptors are examined (SIMON, 1976; PERT & SNYDER, 1976; KLEE & STREATY, 1974; HÖLT et al., 1975). These results do not confirm the theory of COLLIER (1965), explaining tolerance development by an increase of binding sites.

1.7.2. Opiate receptors in the brain, spinal cord and lower medulla

The regional distribution of opiate receptors has been measured after dissection of the brain using the binding assay *in vitro*. A striking regional variation of opiate binding has been observed (PERT & SNYDER, 1973a; KUHAR et al., 1973; HILLER et al., 1973). High levels of receptor binding have been observed for the monkey in the amygdala, periaquaductal grey of the midbrain, medial thalamus and hypothalamus, while binding is not detectable in the cerebellum and white matter. These studies have been followed by *in vivo* labelling attempts using agonists, which have been unsuccessful probably because of the low affinity of the opiate agonists in the presence of high extracellular sodium concentration and the consequently rapid dissociation of the opiate receptor complex (PERT et al., 1976a). Better results have been obtained using the intravenous administration of ^{3}H-diprenorphine, an extremely potent antagonist with a K_D of $1\text{-}2.10^{-10}$M, displaying only a 20% non-specific binding after 1 hr of incubation in the intact animal (PERT et al., 1975). These studies reveal specific locations in distinct nuclei (PERT et al., 1975; ATWEH & KUKAR, 1977a and 1977b). The localization of opiate binding sites in the spinal cord has been published recently (ATWEH & KUHAR, 1977a). A high degree of binding is observed in: layers I (Marginal zone) and II (Substantia gelatinosa) of the dorsal horn of the spinal cord; the substantia gelatinosa of the spinal trigeminal

nucleus; components of the vagal system, including the vagus nerve, nucleus tractus solitarius, nucleus commissuralis, nucleus intercalatus, nucleus ambiguus and nucleus originalis dorsalis vagus; the area postrema. Similar distributions are observed using the potent agonist ^{3}H-etorphine (ATWEH & KUHAR, 1977a). The authors conclude that "in these brain regions, opiate receptors are (1) highly associated with areas receiving small, afferent primary fibers, (2) strategically placed to modulate noxious stimuli as well as explain some visceral side effects of opiate administration" (ATWEH & KUHAR, 1977a).

The opiate receptors are very densely located in areas known to receive primary afferent synapses of small myelinated Aδ and unmyelinated C fibers. Among other sensory modalities, these fibers relay nociceptive information from visceral and somatic organs to higher centers (MELZACK & WALL, 1965; PRICE & MAYER, 1974; DYKES, 1975; KERR, 1975; TREVINO & CARSTENS, 1975). These fibers enter the spinal cord via the dorsal roots and terminate in layer I of Rexed (the marginal zone layer) and layers II and III (substantia gelatinosa) in the dorsal horn (HEIMER & WALL, 1968; COIMBRA et al., 1974; KERR,

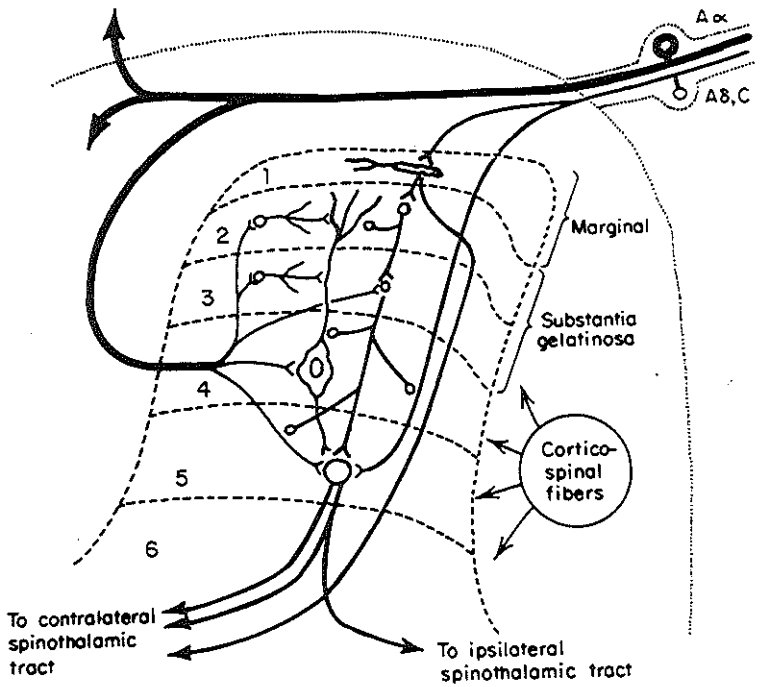


Fig. 1.7.1. Diagrammatic presentation of the layers in the dorsal horn of the spinal cord and of the primary afferent projection into the cord. The numerals indicate Rexed's layers (from HEAVNER, 1975).

1975) where they form a complex association of primary afferents synapsing on dendrites of axons of spinal cells (COIMBRA et al., 1974). Nociceptive stimuli from the face and head terminating via branches of V (trigeminal), VII (facial), IX (glossopharyngeal) and X (vagus) cranial nerves, are carried by unmyelinated C fibers to the spinal tract of the trigeminal nerve (SJÖQUIST, 1938; KERR, 1962 and 1970). These small fibers terminate in the substantia gelatinosa of the spinal trigeminal nucleus, in a manner similar to that described above for the spinal cord (GOBEL, 1973 and 1974).

The exact localization of the opiate binding receptors indicates that at least some of them are located presynaptically since a streak of autoradiographic grains is associated with the vagus nerve and the tractus solitarius (ATWEH & KUHAR, 1977a) both being rich in the strongly labelled C-fibres. Dorsal rhizotomy lowers opiate receptor binding for the monkey dorsal horn (LAMOTTE et al., 1976). The binding is especially affected by lesions in layers I to III where the primary afferent depolarization is located, although the observed decline is only partial. Probably, the opiate receptor is associated with axo-axonic synapses, suggesting a function of the natural ligand of the opiate receptor in the modulation of sensory information at the level of the dorsal horn of the spinal cord (LAMOTTE et al., 1976).

Morphine may have a direct depressant action in the dorsal horn of the spinal cord, though evidence also exists for a mechanism involving the activation by morphine of a descending inhibitory input from higher brain areas (VIGOROUT et al., 1973; PERT & YAKSH, 1974; JAQUET & LAJTHA, 1976). This spinal action of morphine has been demonstrated in numerous studies suggesting a direct blockade (which is reversible by antagonists) on spinal reflexes and spinal transmission following nociceptive stimuli (MATSUSHITA et al., 1971; McCLANE & MARTIN, 1971; CONSEILLER et al., 1972; KRIVOY et al., 1973; JURNA et al., 1973; BESSON et al., 1973; CAVILLO et al., 1974; KITAHATA et al., 1974). In a review BESSON (1976) suggests that morphine may dramatically depress the responses elicited by C-fibre activation. The responses elicited by Aδ-fibre stimulation are less affected, while responses due to Aα-fibres remain unaffected.

In the recent review on the opiate receptor, SNYDER & SIMANTOV (1977) refer to yet unpublished results demonstrating the distribution of the opiate receptor in higher brain regions. Opiate receptors are localized in the solitary nucleus associated with visceral functions, the accessory optic nerve and nuclei, the superior colliculus, the pretectal nuclei and the ventral nucleus of the lateral geniculate body. Within the hypothalamus the opiate receptors are most concentrated in the lateral part of the medial nucleus, in the intralaminar nucleus and the habenular complex. These nuclei are related to the integration of pain sensations. Within the hypothalamus receptors are associated with the infundibulum, connecting the hypothalamus with the posterior pituitary gland.

By using a cellular autoradiographic procedure developed specifically for work with diffusable substances, the localization of ^{3}H-morphine (10 mg/kg, 5 μCi/g body weight) has been studied (WATANABA et al., 1976). The label is associated with the ventromedial hypothalamic nucleus, the medial thalamic nucleus, the tractus opticus, choroid plexus of the lateral ventricle and the adrenal medulla of the rat. Scattered neurons in the cerebral cortex, caudate nucleus, medulla oblongata and spinal cord are also labelled with this pharmacologically relevant dose of morphine. High labelling is also observed in the anterior and posterior pituitary, while the medial lobe is less labelled. Because of the relatively low affinity of morphine for opiate receptors, the resolution of the binding for the opiate receptor using the latter method (WATANABA et al., 1976) seems to be inferior to the method reported by ATWEH & KUHAR 1977a and 1977b).

1.8. OPIOID PEPTIDES AS NATURAL LIGANDS OF THE OPIATE RECEPTOR *IN VIVO*

1.8.1. Identification and distribution of opioid peptides

The selective interaction of opiates with the opiate receptor, its subcellular localization, its regional distribution and the ionic requirements for binding reported in the previous paragraphs suggest that opiate receptors may have a natural ligand in the brain which mimicks the action of opiate agonists. Hughes and coworkers (HUGHES, 1975; HUGHES et al., 1975a) have succeeded in isolating a morphine-like factor, called enkephalin, from brain extracts which has morphine-like properties on two biological preparations, i.e. the inhibition of electrically induced contractions of the mouse vas deferens and the guinea pig ileum. It has a molecular weight of approximately 1000 and loses its biological activity on exposure to proteolytic enzymes. Further purification has indicated that the extracted material contains two morphine-like pentapeptides, i.e. H-TYR-GLY-GLY-PHE-MET-OH (met-enkephalin) and H-TRY-GLY-GLY-PHE-LEU-OH (leu-enkephalin), occurring in a ratio of approximately 3:1 (HUGHES et al., 1975b). By using the ability of opiate agonists to displace radioactively labelled opiates from brain membranes several groups have demonstrated the existence of a morphine-like factor in extracts of brain, pituitary and human cerebrospinal fluid (TERENIUS & WAHLSTRÖM, 1974, 1975a and 1975b; PASTERNAK et al., 1975c; TESCHEMACHER et al., 1975; COX et al., 1975). Subsequent studies indicate that the properties of the morphine-like factor isolated by Pasternak et al. are very similar to those observed for enkephalin as described above, whereas the fraction purified from the pituitary by the group of Goldstein (TESCHEMACHER et al., 1975; COX et al., 1975) has different properties. Upon purification to homogeneity two similar pentapeptides have been identified, viz. met-enkephalin and leu-enkephalin, in calf brain in a ratio of 1:4 (SIMANTOV & SNYDER, 1976). Studies in several species, using displacement of ^{3}H-opiate from the receptor by enkephalin as an assay for this endogenously present opioid peptide, indicate the presence of enkephalin in the brain with a regional distribution very similar to that for the opiate receptor (see Table 1.8.1; HUGHES, 1975; PASTERNAK et al., 1975c and 1976; SIMANTOV et al., 1976c).

Enkephalins have been found mainly in the synaptosomal fraction which may be inaccordance with a transmitter function for these opioid peptides (SIMANTOV et al., 1976b). In opiate receptor assays the material behaves as an opiate agonist (PASTERNAK et al., 1976). Competition of the enkephalins with opiate receptor binding has revealed that leu-enkephalin acts more like a pure agonist than met-enkephalin (SIMANTOV & SNYDER, 1976).

The discovery that met-enkephalin is identical with residues 61-65 of the pitu-

Table 1.8.1.

Regional distribution of enkephalin in monkey brain

Region	*Enkephalin concentration (Units/mg protein)*	*Opiate receptor density* (fmole stereospecific [^3H]dihydromorphine bound/mg protein)*
Cerebral cortex		
Superior temporal gyrus	0.88 ± 0.11	10.8
Inferior temporal gyrus	0.53 ± 0.07	6.0
Postcentral gyrus	0.46 ± 0.05	2.8
Precentral gyrus	0.41 ± 0.07	3.4
Temporal pole	0.36 ± 0.07	
Frontal pole	0.35 ± 0.04	11.9
Occipital pole	0.24 ± 0.04	2.3
White matter areas		
Corpus callosum posterior	0.56 ± 0.05	< 2 (Whole)
Corpus callosum anterior	0.53 ± 0.05	
Optic chiasma	0.40 ± 0.06	< 2
Corona radiata	0.34 ± 0.02	< 2
Limbic cortex		
Amygdala	1.68 ± 0.16	65.1 (Anterior) 34.1 (Posterior)
Hippocampus	0.52 ± 0.07	12.5
Hypothalamus		
Anterior hypothalamus	4.20 ± 0.47	24.3
Posterior hypothalamus	1.35 ± 0.11	24.7
Thalamus		
Medial thalamus	0.57 ± 0.04	24.6
Lateral thalamus	0.25 ± 0.02	7.8
Pulvinar	0.19 ± 0.02	
Extrapyramidal areas		
Head of caudate nucleus	5.28 ± 0.40	19.4
Globus pallidus interior	4.96 ± 0.41	7.7 (Whole globus pallidus)
Globus pallidus exterior	4.92 ± 0.34	
Body of caudate nucleus	3.04 ± 0.37	9.0
Tail of caudate nucleus	1.72 ± 0.20	8.9
Putamen	0.96 ± 0.07	11.7
Midbrain		
Periaqueductal gray	1.48 ± 0.16	31.1
Raphe area	1.08 ± 0.06	8.2
Superior colliculi	0.72 ± 0.05	10.6
Inferior colliculi	0.46 ± 0.03	6.7
Cerebellum- lower brain stem		
Floor of fourth ventricle	1.14 ± 0.08	6.3
Lower medulla oblongata	0.44 ± 0.04	5.8
Deep nucleus	0.17 ± 0.02	
Cerebellar cortex	0.16 ± 0.02	< 2
Spinal cord (cervical)		
Dorsal cord (white and grey)	0.72	3.1 (White)
Ventral cord (white and grey)	0.72	3.3 (White)

The supernatant of a 100,000 x g treated extract of brain regions was assayed for ^3H-naloxone binding inhibition. A unit of enkephalin was defined as that amount of enkephalin which yields 50% receptor occupation (from SIMANTOV et al., 1976a).

itary peptide β-lipotropine (β-LPH) has resulted in the discovery that a β-LPH fragment (β-LPH 61-91) has opioid activity as measured with receptor binding and guinea pig intestine assays (COX et al., 1976b; GRÁF et al., 1976a and 1976b; LI & CHUNG, 1976; LAZARUS et al., 1976; BRADBURY et al., 1976a, 1976b and 1976c).
Very recent reports demonstrate that still more opioid material can be extracted from tissues. TERRENIUS (1976) has reported that the peptides somatostatin and ACTH have partial agonist-antagonist like selectivity for opiate receptors suggesting that they may serve as a natural endogeneous antagonist to the endorphine system. A novel endogeneous analgesic called anodynin has been isolated from human blood (PERT et al., 1976b). Using antibodies against morphine an endogeneous compound reacting with this antibody which inhibits the electrically induced contractions of guinea pig ileum or mouse vas deferens, has been extracted from the brains of many species (GINTZLER et al., 1976). This compound is not a peptide, its action is not antagonized by naloxone and its distribution throughout the brain does not exactly parallel that of the opiate receptor.

1.8.2. A comparison of the properties of opiates and endorphines

The most striking feature of structure-activity studies is the absolute requirement of the aromatic hydroxyl-moiety of tyrosine. Replacement of the N-terminal tyrosine by phenylalanine abolishes opiate receptor binding capacity and the inhibitory effect on guinea pig ileum contractions (CHANG et al., 1976; DAY et al., 1976), while a similar loss of activity is observed after replacement with tryptophan, dihydroxyphenylalanine or the addition of a methylether group to tyrosine (DAY et al., 1976). Removal of the N-terminal NH_2-group also abolishes all activity, while addition of an arginine residue to this N-terminal NH_2-group abolishes biological activity by 50%. The C-terminal part is less critical, because β-LPH (a representative for a substitution of the C-terminal NH_2-group) is slightly less active as compared to met-enkephalin. Peptides smaller than 5 amino acids are all inactive.
The conformation of morphine is essentially defined by the rigidity of its molecular framework. It appears that the tyrosine side chain containing the aromatic hydroxyl-moiety is held in the same direction as the 3-OH group of morphine. The amino-group of enkephalin is in the same position as the tertiary amino group of morphine (BRADBURY et al., 1976a). These authors suggest that the GLY-GLY group forms a β-bend bringing the amino group of phenylalanine near the carboxyl group of tyrosine, which results in the formation of a hydrogen-bond. This allows the phenylethyl group of enkephalin to take the position of the phenylethyl substituent at position 19 of the potent morphine

derivative oripavine (BRADBURY et al., 1976a). The authors suggest that this lipophylic group, present in oripavine and enkephalin, but not in morphine, may contribute to the more potent binding of the first two opiates when compared with morphine. Another group (HORN & RODGERS, 1976) has stressed the importance of the tyramine group present in enkephalin and many opiates incluiding benzmorphan-derivatives. Enkephalin is a flexible molecule which may have a preferential conformation upon binding with the receptor, which may differ from the conformation as suggested from crystallography, NMR spectroscopy or theoretical calculations. It is also worthwhile to note that methadone has no obvious similarity with enkephalin, suggesting that groups other than the tyramine fragment are important for binding. ROQUES et al. (1976) suggest that the TYR-GLY part of the molecule may be involved in the primary attachment of the peptide to the receptor. The relative freedom of the TYR-GLY moiety presumably allows the secondary attachment of the hydrophobic part of the molecule. This model may also account for the relative differences in leu- and met-enkephalin binding.

The notion that the amino acid sequence of met-enkephalin corresponds to residues 61-65 of lipotropin, a pituitary peptide, has resulted in the finding that β-LPH 61-91 is more active than the enkephalins (LAZARUS et al., 1976; BRADBURY et al., 1976c; TSENG et al., 1976; COX et al., 1976b; GRÁF et al., 1976b), although a lower efficacy has also been observed (DAY et al., 1976). It is suggested that the large differences in analgesic action (analgesia is hardly detectable with the enkephalins) is caused by the rapid degradation by proteolytic enzymes *in vivo* (BELLUZI et al., 1976; GRAF et al., 1976b).

Although it is too early to discuss the possible function of β-LPH 61-91 in the brain, certain questions seem relevant, such as: are enkephalins just artefacts formed during isolation by the action of proteolytic enzymes, which normally have no access to the peptides, and which may result in degradation of β-LPH 61-91 and formation of met-enkephalin? (SNYDER & SIMANTOV, 1977). Apparently, negligible amounts of enkephalin are found in the pituitary using radio-immunoassay techniques, although the radio-receptor assay revealed high levels. No differences are observed with both techniques in other brain regions (SNYDER & SIMANTOV, 1977).

1.8.3. Behavioural effects of enkephalins and endorphines

The most important property of met-enkephalin is its analgesic action in the brain, which has been demonstrated recently (although with some difficulty) by several groups (GRÁF et al., 1976b; BÜSCHER et al., 1976; BELLUZZI et al., 1976; CHANG et al., 1976). Partially purified enkephalin is about 100 times more active than synthesized enkephalins in producing analgesia (PERT et al.,

1976b), possibly because impurities may protect enkephalin from proteolytic destruction (SNYDER & SIMANTOV, 1977). However, recently it has been shown that intraventricularly applied met-enkephalin may produce a lowering of the pain threshold upon exposure of the rat to a sustained mildly noxious environment (LEYBIN et al., 1976). These animals when infused with met-enkephalin exhibit signs of withdrawal, viz. wet shakes, rearing, sniffing, restless exploration and increased defaecation, beginning 1 to 3 min after infusion. Similar quasi-morphine abstinence behaviour has been observed after intraventricular injection of met-enkephalin or several β-LPH fragments (BLOOM et al., 1976). After β-LPH administration via the intraventricular route rigidity, loss of righting reflex and absence of spontaneous locomotor activity is observed in these animals. All these effects can be antagonized by administration of naloxone, after which wet shakes are observed (BLOOM et al., 1976).
Chronic administration of β-endorphine elicits physical dependence on the peptides in rats (LOH et al., 1976). Cross-tolerance between met-enkephalin and morphine has been observed *in vitro* using the guinea pig ileum or the mouse vas deferens (WATERFIELD et al., 1976) and it has been demonstrated *in vivo* using the analgesic response in mice and rats (BLÄSIG & HERZ, 1976; VAN REE et al., 1976).

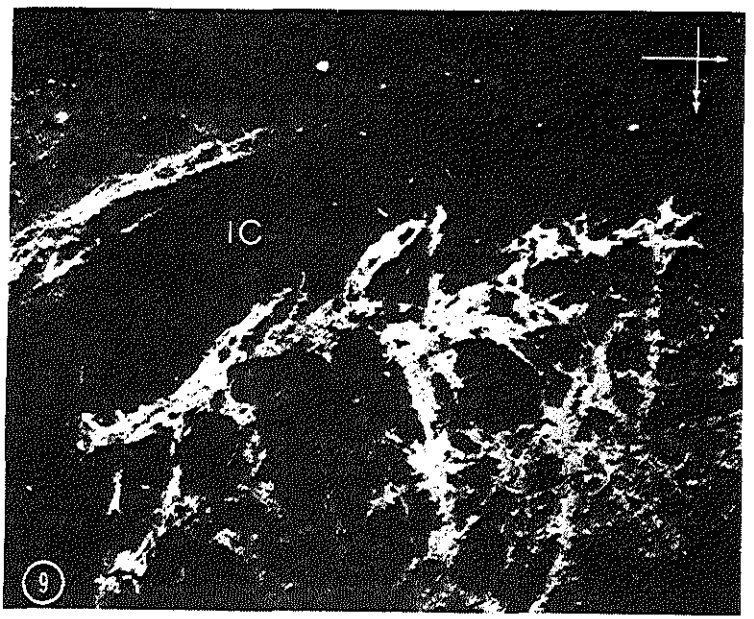


Fig. 1.8.1. Immunofluorescense micrograph of the globus pallidus after incubation with leu-enkepahlin antiserum (from ELDE et al., 1976).

Similar to morphine, met-enkephalin administered via the intraventricular route is capable of inhibiting the morphine abstinence syndrome. Administration of enkephalin immediately before naloxone to morphine dependent mice reduces precipitated withdrawal jumping. Defaecation and rearing behaviour are not inhibited by the administration of both met-enkephalin and morphine (BHARGAVA, 1977a). The latter is 4 times as active as the peptide in inhibiting the stereotyped withdrawal response to morphine in dependent mice.
Experiments performed by DE WIED and co-workers (VAN REE & DE WIED, 1976; DE WIED, 1977a) indicate that enkephalin may have other functions. It has been observed that subcutaneous injection of met-enkephalin in extremely small amounts (in the order of 100 ng/kg) inhibits the extinction of conditioned avoidance responding, an effect also observed with ACTH 4-10. ACTH 4-10 is present in ACTH itself, but also in α-MSH, β-MSH and β-LPH (viz. β-LPH 47-53). This indicates that two behaviourally very active peptides may be derived from the common precursor poly-peptide β-LPH, "the N-terminal part producing the precursor for neuropeptides involved in motivational, learning and memory processes, while the C-terminal part of this molecule is involved in opiate-like activities" (DE WIED, 1977b).

1.8.4. Enkephalin as a possible neurotransmitter candidate

When morphine is applied iontophoretically to single neurons in the rat brain stem, both excitatory and inhibitory effects are observed (BRADLEY & DAY, 1974), the former effect being sensitive to the morphine antagonist naloxone and displaying stereoselectivity (BRAMWELL & BRADLEY, 1974; SATOH et al., 1974). A physiological role for met-enkephalin is suggested by the finding that it depresses 70% of the spontaneously active neurons tested, whereas 11% are excited. All neurons depressed by morphine are similarly depressed by met-enkephalin, while naloxone inhibits the inhibitory effect of met-enkephalin. Similar results have been obtained in the cat, although naloxone is not able to reverse the effect of the peptide (GENT & WOLSTENCROFT, 1976). Spontaneous and glutamate-induced firing is depressed by enkephalin in a naloxone-revesible manner in many brain regions containing enkephalin and opiate receptors in high concentration, viz. frontal cortex, caudate nucleus and peri-aquaductal grey (FREDERICKSON & NORRIS, 1976).
Thalamic neurons, excited by noxious stimuli and presumably nociceptive in nature, are very sensitive to met-enkephalin and can be suppressed for 12 min after the end of the application of the peptide, while only few are sensitive to naloxone (HILL et al., 1976). In the spinal cord painful stimulation-evoked responses of neurons in the substantia gelatinosa are suppressed by morphine and enkephalin in a naloxone reversible manner (DUGGAN et al., 1976). More

consistent effects are observed with met-enkephalin-amide, where a recovery time of 10 min is observed. When tested on Renshaw cells only excitation is observed with morphine and met-enkephalin, which is sensitive to naloxone (DAVIES & DRAY, 1976).

The aforementioned results suggest a neurotransmitter or neuromodulator role for met-enkephalin in the brain and spinal cord. Immunohistochemical studies using antibodies to leu-enkephalin reveal a widespreas system of axons and their terminals in the rat nervous system (ELDE et al., 1976). In the medulla oblongata highest fluorescent fibres are observed in the N. reticularis lateralis, N. ambiguus, N. tractus solitarius, N. originalis nervi facialis and N. originis nervi hypoglossi. At the pontine level dense networks are observed in the central grey and N. parabrachialis dorsalis. At the mesencephalic level an intense reaction is observed in the periaquaductal grey and the zona compacta of substantia nigra, as well as in the medial lemniscus. Most hypothalamic nuclei are positively marked, especially the ventro- and dorso-medial nuclei, the periventricular area and the medial preoptic nucleus. In the thalamus the N. paratenialis is densely packed with labelled fibres. In the telencephalon a very profuse and intense immunofluorescense is observed in the globus pallidus (see Fig. 1.8.1). The rostral and ventral parts of the caudate contain the most labelled fibres. Dense fibres are further observed in the lateral portion of N. interstitutialis stria terminalis, N. accumbens, and N. amygdaloideus centralis, while other amygdaloidal nuclei contain variable labelling. No labelling is observed in the cortex and pituitary. These results of ELDE et al. (1976) are closely parallel to the results found in the monkey demonstrating the distribution in brain regions of endogenous enkephalins (SIMANTOV & SNYDER, 1976; see also Table 1.8.1.) and are in accordance with other localization studies of the opiate receptor (HILLER et al., 1973; KUHAR et al., 1973; PERT et al., 1975; ATWEH & KUHAR, 1977a and 1977b). A lack of correlation is the negative finding in the cortex where opiate receptors are numerous. Enkephalin levels may be low in this region, or, alternatively, other endorphines may be prominent in this area (ELDE et al., 1976). From these studies it is tempting to suggest that enkephalin may fulfil a neurotransmitter function or neuromodulator function in the brain (KOSTERLITZ & HUGHES, 1975). The enkephalins may act as an inhibitory transmitter in the brain exerting a post-synaptic or presynaptic inhibitory effect, or they may be the compounds responsible for interneuron modulation, viz. an inhibitory effect on inhibitory interneurons. A model for the development of tolerance and physical dependence based on the above mentioned mechanisms has been presented (KOSTERLITZ & HUGHES, 1975; SNYDER & SIMANTOV, 1977).

1.8.5. Opioid peptides as partial agonist-antagonists

The enhancement of antagonist binding and the diminisment of agonist binding by incubation in the presence of 50 to 100 mM NaCl has been discussed in chapter 1.7. This selective effect of NaCl on opiate binding correlates well with the properties of the opiates. Pure agonists have a ratio of EC_{50} values with and without NaCl of 12 to 60, while pure antagonists have a ratio of 0.2 to 1.0. Partial agonist-antagonists have a ratio between 1.5 and 12 (PERT & SNYDER, 1975). Experiments with natural "enkephalin" give a value of 6, indicating that "enkephalin" is a relatively pure agonist with only minor antagonist properties (PASTERNAK et al., 1976). Comparable results have been obtained by CHANG et al., (1976). A sodium ratio of 9 has been found for met-enkephalin, while for β-LPH 61-91 a ratio of 1.3 is observed (BRADBURY et al., 1976c). Using the

Table 1.8.2.

The sodium ratio for opiates and opioid peptides

opioid	sodium ratio for agonist/antagonist binding		
	PERT & SNYDER	CHANG et al.	BRADBURY et al.
pure agonists	12-60	50	–
pentazocine	3.3	4	–
pure antagonists	0.2-1.0	–	–
enkephalin	6	–	–
met-enkephalin	3.75	5	9
leu-enkephalin	6.7	14	–
β-LPH 61-91	–	–	1.3

(from: PERT & SNYDER, 1975; CHANG et al., 1976; BRADBURY et al., 1976c)

potency ratio for opiates to displace radioactively labelled and bound agonists and antagonists respectively, a ratio of 11 is observed for met-enkephalin (comparable to the value of 5-6 found for agonists), while β-LPH has a ratio of 2.2 (more similar to the value of 0.7 for the antagonists naloxone) (BRADBURY et al., 1976c). The ratio of ED_{50} with and without NaCl is 3.75 for met-enkephalin and 6.7 for leu-enkephalin, suggesting that met-enkephalin is a partial agonist-antagonist (SIMANTOV & SNYDER, 1976) having a ratio comparable with that of pentazocine, viz. 3.3 While confirming some of the results of

SNYDER's group, the results of Bradbury and co-workers with the potency ratio for antagonist and agonist binding displacement together with the results on the NaCl response suggest that β-LPH may act like an antagonist. These results obviously raise the question whether these findings are compatible with the potent analgesic effect measured after β-LPH 61-91 administration, which is generally considered as an agonistic effect of opiates.

The finding that met-enkephalin behaves like a narcotic antagonist under certain conditions (the presence of a painful stimulus) and the observation that met-enkephalin and β-LPH 61-91 may elicit quasi-morphine abstinence behaviour clearly illustrates the complicated nature of the endogeneous opioid peptides. Of considerable importance in this respect may be the observation that met-enkephalin produces hyperalgesia and withdrawal-like symptoms in the rat during a painful stimulus, while leu-enkephalin produces analgesia under these conditions (LEYBIN et al., 1976). The observation that high doses of pentazocine – a partial agonist-antagonist with a sodium ratio similar to met-enkephalin – may provoke head and body shakes in naive rats (SCHNEIDER, 1968) suggests that quasi-morphine abstinence behaviour may be a common property of partial agonist-antagonists (BLÄSIG et al., 1976).

1.9. INTERACTIONS BETWEEN THE GABA-ERGIC SYSTEM AND OPIATES OR OPIOID PEPTIDES

A growing number of reviews is now available summarizing the interactions between neurotransmitter systems, such as the dopaminergic, cholinergic, noradrenergic or serotonergic system, and the acute and chronic effects of opiates (TAKEMORI, 1974, 1975 and 1976; LAL, 1975; DOMINO et al., 1976a and 1976b; EIDELBERG, 1976). In this chapter the relatively poor evidence is summarized indicating possible specific interactions between GABA and the acute or chronic effects of opiates.

1.9.1. GABA and acute effects of opiates or opioid peptides

The implication of GABA in the effects observed after acute administration of opiates has been suggested by COSTA et al. (1975a). They argued that "the action of morphine on dopaminergic and cholinergic mechanisms in N. caudatus could be explained by assuming that morphine prevents the release of GABA at two possible sites of action: at presynaptic sites on dopaminergic nerve terminals and in the neuronal loop that controls excitability of dopaminergic cell bodies by feed-back inhibition." LAHTI & LOSEY (1974) have demonstrated that pretreatment with the GABA-transaminase inhibitor AOAA antagonizes the morphine or chlorpromazine induced increase of dopamine decline after treatment with the dopamine-synthesis inhibitor α-methyl-p-tyrosine. AOAA itself has no effect on the decline of dopamine after synthesis inhibition.
Administration of morphine reduces the level of c-GMP in cerebellum at a dose of 1 mg/kg as measured after 15 min (KATZ & CATRAVAS, 1976). On the other hand, accumulation of c-GMP has been observed in striatum of rats after treatment with 10 to 30 mg/kg morphine. The increase of c-GMP is maximal after 5 min using 20 mg/kg morphine, and could be antagonized by naltrexone, a morphine antagonist (RACAGNI et al., 1976). Under these conditions no change is observed in the content of c-AMP. The concentration of c-GMP in cerebellum, but also in striatum, increases after inhibition of GABA-synthesis by the glutamate decarboxylase inhibitor isoniazid. Therefore, the observed increase of c-GMP by morphine may be explained by a direct or indirect inhibition of GABA-ergic transmission (BIGGIO & GUIDOTTI, 1976). The present data on the effect of morphine and GABA on the c-GMP content in striatum and cerebellum do not exclude the possibility that morphine decreases GABA-ergic activity in these brain areas.
Exposure to pain (100 sec on a hot plate of 60°C) increases GABA and the ratio of GABA and glutamate concentration in cortical sections of the rat brain, while glutamate concentration is decreased (SHERMAN & GEBHART, 1974). These

changes are prevented by 3 mg/kg morphine sulphate, whereas restraint stress decreases GABA and glutamate without affecting the ratio of both. In a further study in mice consistent effects are observed using pain and stress exposure in combination with morphine (3 mg/kg) and naloxone (SHERMAN & GEBHART, 1976). Although the authors state that the level of glutamate may be a useful central neurobiochemical indicant of exposure to pain, specific effects have also been observed on the GABA-ergic system. In the midbrain reticular formation, GABA levels decrease after pretreatment with morphine alone, or together with pain exposure, in a naloxone-reversible manner. Furthermore, pain exposure increases GABA concentration in central grey and hypothalamus, where morphine alone has no effect on GABA levels. This pain-induced increase of GABA concentration is partially blocked by morphine in a naloxone-reversible manner in central grey and is completely blocked in the hypothalamus, although not in a naloxone reversible manner. It is concluded that specific, regionally localized, effects of morphine on the GABA-ergic system may be detectable using low, analgesic doses of morphine. It must be noted in this respect, that the possibility of post-mortem effects and compartmentation-complications may arise in studies of this type (TAPPAZ et al., 1977).

The involvement of GABA in the analgesic action of opiates is further demonstrated by the following experiments. AOAA increases the analgesic action of morphine, whereas bicuculline and semicarbazide, an inhibitor of GABA-synthesis, decreases it (YONEDA et al., 1976). This finding is in conflict with the observation of WAY (1973) that GABA and AOAA antagonize the analgesic action of morphine. A potentiation by AOAA and p-chlorophenyl-GABA (lioresal, baclophen) has been observed on the analgesic action of methadone, while the methadone induced catatonia was not affected (KÄARIÄNEN & VIKING, 1976). These partially conflicting results may arise from the low specificity of AOAA in high dosage, which may have opposite effects on different GABA compartments. In low doses, it probably inhibits GABA-transaminase selectively (see further), while higher doses may have additional effects on GABA synthesis.

1.9.2. Effects of GABA and opiates or opioid peptides on the cellular level

The cell-firing of many spinal neurons is inhibited by morphine and these neurons are also inhibited by iontophoretically applied GABA. However, far more GABA is needed for such blockade of cell-firing as compared to the effective currents of glutamate, aspartate or glycine on identical neurons (DOSTROVSKY & POMMERANZ, 1973). It has been suggested that the action of met-enkephalin is very similar to the effect of GABA on cell-firing, exhibiting a rapid onset and offset of action. However, in the same study it is demonstrated

that prolonged application of met-enkephalin depresses cell-firing up to 12 min (HILL et al., 1976). Tonically active morphine and enkephalin sensitive interneurones may exist within the periaquaductal grey matter (YAKSH et al., 1976). Opiate action, depressing such an interneuron, may disinhibit a second order neuron providing ascending and descending modulation of sensory transmission (FREDERICKSON & NORRIS, 1976). Suggestions as indicated above are highly speculative and hardly based on real findings. Apparently, no solid evidence has yet been obtained that may implicate a role of the GABA-ergic system in the action of opiates or opioid peptides on cell-firing.
SNYDER & SIMANTOV (1977) summarizing the available data about a presynaptic localization of the opiate receptor in many brain regions suggest that enkephaline neurons may form axo-axonic synapses and mediate pre-synaptic inhibition in some parts of the brain. In other brain areas axo-dendritic or axo-somatic synapses may be associated with post-synaptic inhibition. They conclude that "precedent for both types of inhibition involving a single neurotransmitter exists for GABA neurons, which play major roles in both pre-synaptic and post-synaptic inhibition", suggesting that somehow opiates and opioid peptides may act via GABA-ergic neurons (SNYDER & SIMANTOV, 1977).

1.9.3. GABA and chronic treatment with opiates

The development of tolerance and physical dependence on morphine can be accelerated by the treatment with c-AMP and tryptophan (WAY, 1973; TAKEMORI, 1975). Also, GABA accelerates the development of tolerance and physical dependence (WAY, 1973). The GABA-transaminase inhibitor AOAA is much more effective in this respect, while an inhibition of the development of tolerance and physical dependence is observed after the treatment with the GABA antagonist bicuculline (WAY, 1973; HO et al., 1976). Moreover, HO et al. (1976) have recently demonstrated differential effects of β-alanine and 2,4-diaminobutyrate on the development of tolerance and physical dependence, implicating GABA uptake systems of glial or neuronal origin in these phenomena. Dependence development is accelerated by β-alanine administration. However, the effect of β-alanine may be also explained by an effect of β-alanine on the expression of dependence, viz. stereotyped jumping after administration of naloxone to dependent mice. The treatment with 2,4-diaminobutyrate, inhibiting neuronal GABA-uptake, has no effect on dependence development, but it inhibits the analgesic action of morphine. The development of tolerance is enhanced, but the effect is not statistically significant (HO et al., 1973a and 1973b; HO & LOH, 1974; HO et al., 1976). Increased levels of GABA have been described in rats rendered tolerant to morphine by a multiple injection schedule (LIN et al., 1973).

In these studies dependence development has been quantified, using the method of WAY et al. (1969), as the ED_{50} for stereotyped platform-jumping after injection of naloxone in dependent mice. This method has been criticized especially where groups of animals with different degrees of dependence are compared (see chapter 1.6 for more details). Therefore, the results on dependence development, where different degrees of dependence of treated versus control groups are compared, have to await further confirmation using other species with more abstinence signs being noted. The experiments with AOAA and bicuculline on tolerance development are less doubtful, especially because bicuculline is a rather specific GABA antagonist. These latter experiments therefore demonstrate, that the GABA-ergic system is involved in the chronic effects of morphine. Some confirmation for these observations is provided by the report demonstrating that a vitamin B_6 deficient diet inhibits the development of physical dependence in rats measured as the decrease in wet shaking frequency after naloxone injection in the dependent animals (SIU et al., 1976). The frequency of wet shaking decreased from 6-10 shakes per 10 min to 1-2 per 10 min. Because the activity of glutamate decarboxylase is decreased during vitamin-B_6 deficiency (BAYOUMI et al., 1972), an unimpaired synthesis of GABA seems to be a prerequisite for the development of physical dependence or, alternatively, for a full expression of the wet shakes.

1.10. CALCIUM AND THE ACTION OF OPIATES ON THE CELLULAR LEVEL

Calcium ion is important as a membrane stabilizer and as a membrane constituent, but above all it is important because the fluxes of calcium across the cell membrane are associated with depolarization of neurones and neurotransmitter release. Calcium administration antagonizes the analgesic action of morphine, the level of calcium in the brains of mice decreases after injection of morphine and calcium administration inhibits the development of tolerance and physical dependence (KANETO, 1971). Treatment with the Ca^{2+}-chelator EGTA potentiates the actions of opiates, while the Ca^{2+}-antagonist La^{3+} and Ce^{2+} also have potentiating effects on the actions of opiates (HARRIS et al., 1975 and 1976). Therefore, the action of calcium ion is important for both the acute and chronic effects of morphine.

Recent studies indicate that the calcium loss observed after acute administration of opiates is localized in the nerve-ending fraction (ROSS et al., 1976). The 50% loss from the nerve ending fraction is paralleled by a 25% increase in the calcium content of free mitochondria. The effect of morphine on calcium levels in nerve endings is prevented by naloxone, whereas the increase in calcium content of free mitochondria is not. It is concluded that morphine affects a calcium pool located in synaptosomes via a naloxone reversible mechanism.

Studies of the binding of ^{45}Ca reveal that chronic treatment with morphine increases the amount of ^{45}Ca in all nerve ending-containing fractions. Further studies indicate that the Ca-effect is located in intrasynaptosomal vesicles or Ca-binding intrasynaptosomal mitochondria. Acute treatment with morphine decreases this Ca-binding, while during naloxone precipitated abstinence elevated Ca-levels rapidly decline to control values within 15 min (HARRIS et al., 1977). These experiments suggest that opiates may inhibit the release of a variety of neurotransmitters via Ca^{2+}-depletion. The increase of calcium observed during tolerance development during chronic opiate treatment may reduce the inhibitory effect of narcotics on neurotransmitter release resulting in tolerance (HARRIS et al., 1977). It is interesting to note that the calcium antagonists La^{3+} and Ca^{2+} in high doses may cause analgesia – an opiate agonist effect – and exhibit cross-tolerance with morphine (HARRIS et al., 1976; YAMAMOTO et al., 1976). Similar results as for ^{45}Ca binding studies have been obtained by measuring the level of Ca during tolerance development. A 60% increase of synaptosomal calcium is observed 72 hrs after pellet implantation (ROSS et al., 1976).

Calcium administration (45 mg/kg) 1 hr before administration of naloxone to morphine dependent rats inhibits several signs of abstinence, including wet shaking, ptosis, defaecation and diarrhoea (SANGHVI & GERSHON, 1976). This observation is probably associated with the observed return to control

levels of calcium after naloxone administration to morphine dependent rats as discussed above.

In conclusion, these observations implicate Ca^{2+} as an important factor in the acute and chronic effects of opiates. It is clear that many secondary effects may arise from changes in the distribution of Ca^{2+}, since it is involved in the regulation of many phenomena associated with the events on the cellular level, such as release of transmitter substances, regulation of adenylate cyclase, guanylate cyclase and many other enzymes, nerve impulse flow etc.

1.11. QUASI-MORPHINE ABSTINENCE BEHAVIOUR

1.11.1. Definition of quasi-morphine abstinence behaviour and its implications

"Quasi-morphine abstinence behaviour" has been defined by COLLIER (1974) as "an effect resembling one elicited by withdrawal of a drug on which an animal has been made dependent, but produced by another treatment in a naive animal never exposed to drug nor to a like-acting congener that induces such dependence". It must be recognised that the usefulness of such an effect, produced other than by exposure to a drug of dependence, for the study of dependence mechanisms relies on analogy. The implication of such a system in the development of dependence strengthens the value of the model. Therefore it has been argued that quasi-morphine abstinence effects can be a starting point of the study of dependence mechanisms.
Theophylline, a phosphodiesterase inhibitor, induces head shakes, body shakes, diarrhea, weight loss and irritation to touch, effects which also form part of the morphine abstinence syndrome (COLLIER, 1974; COLLIER et al., 1974). Theophylline increases the incidence of many symptoms, the combination of theophylline and naloxone (10 mg/kg) closely resembles the true morphine abstinence syndrome, while the effect of theophylline is blocked by 1 mg/kg heroin in a naloxone reversible manner. Apparently, the treatment with theophylline resembles the true morphine abstinence syndrome in four ways: firstly, many of the same responses increase in incidence; secondly, naloxone increases the incidence of symptoms; thirdly, heroin lessens the incidence of responses; and fourthly, naloxone antagonizes this effect of heroin.

1.11.2. Quasi-morphine abstinence behaviour after injection of various compounds

Quasi-morphine abstinence behaviour may occur after the treatment with varying compounds: thyrotropin-releasing hormone, injected into the brain (PRANGLE et al., 1974; WEI et al., 1975c); AG-3-5 (WEI et al., 1975c; WEI, 1976); RX 336-M (COWAN & McFARLANE, 1976); benzylideneaminooxycarbonic acid derivatives (JAHN & MIXICH, 1976); stimulation of the medial thalamus (FREDERICKSON et al., 1975). It has also been suggested that 5-hydroxytryptophan in combination with a peripheral decarboxylase inhibitor and other treatments increasing the central serotonergic activity may induce shaking behaviour (BEDARD & PYCOCK, 1976). Until the effects of morphine are studied the relevance of this finding remains to be established.
Recent observations after intracerebral administration of opioid peptides relate shaking behaviour in rats with the acute effect of endorphines like met-enke-

phalin and a number of β-LPH 61-91 fragments (BLOOM et al., 1976; LEYBIN et al., 1976). This behaviour has been associated with the partial agonist-antagonist properties of these opioid peptides as discussed in chapter 1.8. The sustained pain stimulation may result in opioid peptide release, and it has been suggested that met-enkephalin may precipitate acute abstinence by acting as an antagonist under such conditions (LEYBIN et al., 1976). These observations may be related to the finding that high doses of pentazocine – a partial agonist-antagonist with a sodium ratio similar to met-enkephalin – may provoke head and body shakes in naive rats (SCHNEIDER, 1968). Pentazocine is of interest since it is a relatively non-addictive analgesic with possible clinical relevance. Furthermore, it is similar to met-enkephalin in its relative agonist-antagonist properties.

2. BIOCHEMICAL SECTION

2.1. MATERIAL AND METHODS

2.1.1. The measurement of glutamate decarboxylase activity

Tissue preparation. Male Wistar random rats (125-175 g) were killed by decapitation and their brains were rapidly removed, cooled on ice-water and weighed. A 10% w/v homogenate was prepared by blending the brain in a Teflon-glass Potter-Elvehjem homogenizer with an ice-cold solution of 0.32 M sucrose and 4.5 mM 2-mercaptoethanol. A small portion of the homogenate was kept apart for protein measurements. One vol of homogenate was added to 3 vol of ice-cold Triton medium (0.67% w/v Triton X-100, 20 mM 2-mercaptoethanol and 50 mM KH_2PO_4/K_2HPO_4-buffer, pH 6.5) and kept on ice-water for 1 h before use.
Radiochemical procedure. Glutamate decarboxylase activity was estimated using a radiochemical method which is based upon the production of radioactive $^{14}CO_2$ from 1-^{14}C-L-glutamate (ROBERTS & SIMONSEN, 1963). Assays were performed in vessels especially equiped for these experiments (Fig. 2.1.1.).

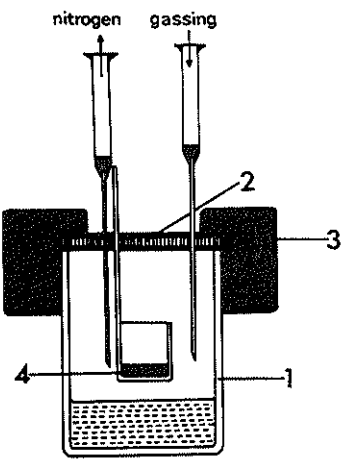


Fig. 2.1.1. Special vessels for the glutamate decarboxylase assay. Vessels (1) containing the incubation mixture were sealed by septum (2), which was tightly pressed between the top of the vessel and a cap (3) that could be screwed onto it. Through a central spare in the cap a small vessel (4) containing hyamine 10-X-hydroxide (dried overnight to prevent overdistilling during incubation of methanol present in the hyamine solution) was inserted.

Gassing by nitrogen could be performed through two removable injection needles penetrating through the septum into the vessel. The $^{14}CO_2$ produced was trapped in the hyamine base so that radioactive ^{14}C could be measured. All solutions were gassed before use with nitrogen for 10 min. Gassing was also performed with the homogenate to remove endogenous oxygen traces present in the brain tissue.

Glutamate decarboxylase assay. Unless otherwise specified, the incubation mixture contained: 50 μmoles KH_2PO_4/K_2HPO_4-buffer, pH 6.5; 9.0 μmoles L-glutamate; 0.4 μmoles PLP; 20 μmoles 2-mercaptoethanol; 0.5 μCi DL-1-^{14}C-glutamatic acid (spec. act.: 29 mCi/mMol) and 0.5 ml of Triton treated homogenate equivalent to 12.5 mg tissue (wet wt), in a total vol of 1.0 ml. After addition of all components of the incubation mixture except glutamate, the incubation vessels were gassed for 10 min with nitrogen. After 2 min of preincubation at 38°C the reaction was started by the injection of 0.2 ml 45 mM L-glutamate solution, containing 2.5 μCi DL-1-^{14}C-glutamic acid, 50 mM KH_2PO_4/K_2HPO_4-buffer, pH 6.5, and 20 mM 2-mercaptoethanol. The incubations were performed in a Dubnoff shaking incubator for 60 min at 38°C. The reaction was stopped by the injection of 0.1 ml 4 M H_2SO_4 solution. After standing overnight at room temperature to allow absorption of the radioactive $^{14}CO_2$, the hyamine containing vessels were put into disposable counting vials containing 10 ml scintillation solution (60 g naphtalene, 4 g 2,5-diphenyloxazole, 0.2 g 1,4-bis-2-(5-phenyloxazolyl)benzene and dioxan in a total volume of 1 liter. After standing for an hour to allow dissolution of the hyamine, the radioactivity in the vials was measured in a Packard TriCarb liquid scintillation spectrometer model 3375. From the specific radioactivity (expressed as dpm/μmole) of the original glutamate solution containing 45 mM glutamate and from the amount of radioactivity in dpm, trapped in the hyamine vessel, the amount of CO_2 produced was calculated using the formula:

$$CO_2 \text{ production} = \text{dpm trapped} \cdot 2 / \text{spec. radioact. of glutamate}$$

The production of CO_2 is expressed as μmoles/ml. The factor 2 was introduced because only 50% of the radioactive sample consists of 1-^{14}C-L-glutamate, participating in the decarboxylation reaction, while the other 50%, consisting of the D-isomer, does not participate (ROBERTS & SIMONSEN, 1963). The results were not corrected for the trapping efficiency of the hyamine vessels. For all experiments blanks were run in parallel, containing tissue that was heated for 5 min in a boiling waterbath. These blanks were carried through the entire assay procedure. Glutamate decarboxylase activity was expressed as μmoles/g tissue (wet wt)/h or as μmoles/100 mg protein/h.

2.1.2. Complex formation with PLP

Complex formation with PLP was measured by following the decrease in absorbance of the characteristic 385 nm peak in the spectrum of PLP. This peak corresponds with an intact formaldehyde group at pH 6.5. Interaction with the formaldehyde group results in the disappearance of the peak at 385 nm, which is responsible for the yellow colour of PLP (BLACK & AXELROD, 1969). The peak present at 325 nm increased concurrently in height. The absorbance of PLP was measured using a Beckman model DB-G grating spectrophotometer with scanning equipment. The sample cuvette (volume: 1 ml, optic pathway: 1 cm) contained: 50 mM KH_2PO_4/K_2HPO_4-buffer, pH 6.5; 20 mM 2-mercaptoethanol; 0.5 mM PLP and in some cases Triton treated homogenate as used in the glutamate decarboxylase assay. The control cuvette contained a similar mixture without the PLP. Drugs were introduced in both cuvettes, quickly mixed and the extinction at 385 nm was recorded. All reactions were performed at 38°C. The time course of the disappearance of the extinction at 385 nm was plotted semilogarithmically as described further on, and the rate of complex formation was calculated from the obtained straight lines.

2.1.3. Protein measurement

Protein was measured as described by LOWRY et al. (1951) using bovine serum albumine as a standard.

2.1.4. Measurement of GABA-transaminase activity

Tissue preparation. A 10% w/v homogenate in 0.32 M sucrose was prepared as described in 2.1.1. One volume of the homogenate was added to 3 volumes of an ice-cold Triton X-100 medium (0.67% w/v Triton X-100, 50 mM Tris-HCl, pH 8.5, and 4.5 mM 2-mercaptoethanol) and was kept on ice-water for 1 h before use.

Fluorimetric GABA-transaminase assay. For the assay of GABA-transaminase a coupled enzyme system was used in which GABA is converted into SSA and subsequently oxidized by SSA-dehydrogenase into succinate. During the latter reaction NAD is converted into NADH, which was determined fluorimetrically. The incubation mixture contained: 50 mM Tris-HCl, pH 8.5; 3.0 mM GABA; 2.0 mM 2-oxoglutaric acid; 20 mM 2-mercaptoethanol; 0.9 or 1.1 mM NAD and 0.2 or 0.3 ml of Triton-treated homogenate in a total volume of 1.13 ml. After preincubation for 30 min in the absence of GABA at 22°C and subsequent cooling on ice-water, 0.1 ml of 33.75 mM GABA was added to the ice-cold in-

cubation mixture and the reaction was started by placing the tubes at room temperature (22°C). The incubations were performed in duplicate. The reaction was stopped after the appropriate incubation time (in general 2 h) by placing the tubes in ice-water. NADH formation was measured as described by LOWRY et al. (1975) with the following modifications: triplicate samples of 0.1 ml taken from the ice-cold incubation mixture were carefully mixed with 0.1 ml of ice-cold phosphate buffer (0.35 M sodium phosphate, pH 11). After heating for 15 min at 60°C to destroy excess of NAD, a highly fluorescent product was obtained by a second incubation period at 60°C in the presence of an additional 1.3 ml of 7.5 M NaOH containing 0.02% w/v H_2O_2. Fluorescence was measured after dilution with 3.0 ml water using excitation at 370 nm (uncorrected) and measuring at 455 nm in a Baird Atomic spectrophotofluorimeter (Fluorespec or Fluoripoint), the latter equiped with a flow cell and an automatic sampling and reading unit). Standards containing NADH or buffer in addition to the incubation mixture were carried throughout the whole assay procedure except the incubation period. NADH was standardized daily in 1.0 mM Na_3PO_4 solution (molar extinction: 6270 M^{-1}), as described by PITTS et al. (1965). The results were corrected for tissue and substrate blanks by omitting GABA, tissue or both from the incubation mixture. Some initial experiments were performed with slightly different incubation conditions. The incubation mixture contained 0.6 ml of Triton-treated homogenate in a total volume of 2.25 ml, while the reaction was started without preincubation by placing the complete incubation mixture at 22°C. The results were corrected for NADH formation in blanks containing the complete incubation mixture and in addition 0.045 mM AOAA. Under these conditions GABA-transaminase is completely inhibited. Activity was expressed as μmol/g (wet wt)/h or μmol/100 mg protein/h.

Radiochemical GABA-transaminase assay. This method is based on the conversion of ^{14}C-GABA into ^{14}C-SSA and ^{14}C-succinate by GABA-transaminase and SSA-dehydrogenase. Substrate and products are separated from each other by DOWEX chromatography. The incubation mixture contained: 50 mM Tris-HCl, pH 8.5 ; 3.0 mM GABA and 0.09 μCi (U-^{14}C)-GABA (spec. act. 4.6 mCi/mMol); 2.0 mM 2-oxoglutaric acid; 20 mM 2-mercaptoethanol; 0.9 mM NAD; 1.0 mM sodium succinate and 2.22 mg tissue/ml. After a 30 min preincubation period at 22°C in the absence of GABA, the reaction was started by the addition of the labelled GABA to the incubation mixture. After a 120 min incubation period the reaction was stopped by placing the tubes in ice-water (viz. similarly as described for the fluorimetric GABA-transaminase assay) and 1 vol of ice-cold 8% trichloroacetic acid was added. After centrifugation for 10 min at 3000 rpm 0.5 ml of the supernatant was brought on a Dowex 50 WX-4 (200-400 mesh) column and analyzed for the presence of acidic metabolites (^{14}C-succinate and ^{14}C-SSA) and ^{14}C-GABA as described by HALL & KRAVITZ (1967). Blanks contained 0.045 mM AOAA in addition to the complete incubation mixture.

The activity of GABA-transaminase was calculated from the percentage conversion of ^{14}C-GABA into ^{14}C-SSA and ^{14}C-succinate.

2.1.5. Measurement of SSA-dehydrogenase activity

Fluorimetric assay of rat brain SSA-dehydrogenase. SSA-dehydrogenase activity was estimated as described by KAMMERAAT & VELDSTRA (1968) with some modifications by measuring NADH formation from NAD using SSA as a substrate. A concentrated solution of SSA was prepared from monosodium glutamate (KAMMERAAT, 1966). After dilution SSA was completely converted into succinate by SSA-dehydrogenase using standardized assay conditions as described below. The concentration of SSA was calculated from NADH production after correction for tissue and reagent blanks.
The incubation mixture contained: 50 mM Tris-HCl, pH 8.5; 0.25 mM NAD; 0.3 mM SSA; 8.2 mM 2-mercaptoethanol and 0.2 ml of Triton X-100 treated homogenate (see above) in a total vol of 1.13 ml. After preincubation for 30 min at 22°C in the absence of SSA, 0.1 ml of 3.375 mM SSA was added to the ice-cold incubation mixture and the reaction was started by placing the tubes at 22°C. The incubations were performed in duplicate. The reaction was stopped after the appropriate incubation time by placing the tubes in ice-water. NADH production was measured exactly as described for GABA-transaminase. The results were corrected for tissue ans substrate blanks by omitting SSA, tissue or both from the incubation mixture. Activity was expressed as μmol/g tissue wet wt/h or as μmol/100 mg protein/h.
Spectrophotometric assay of bacterial SSA-dehydrogenase. Partially purified bacterial SSA-dehydrogenase prepared from *pseudomonas fluorescence* (Worthington, "GABASE") was used for these experiments without further purification. SSA-dehydrogenase activity was measured using a Beckman spectrophotometer equiped with a recorder. The enzyme preparation was dissolved in a medium containing 0.225 vol of 0.32 M sucrose, 0.025 vol of 300 mM 2-mercaptoethanol and 0.75 vol of 0.67% w/v Triton X-100 in 50 mM Tris-HCl, pH 8.5, and kept in ice-water before use. The reaction mixture contained 50 mM Tris-HCl, pH 8.5; 10 mM 2-mercaptoethanol; 0.5 mM NADP; 0.44 mg GABASE per ml and SSA. The experiments were performed at 38°C. The cuvettes contained the complete incubation mixture except substrate at the start of the experiment. After a 2 min incubation period the reaction was started by the addition of substrate to the sample cuvette and buffer to the reference cuvette. The time course of the reaction was followed for 5 min by measuring the rate of NADPH formation from NADP, measured as the change in extinction at 340 mm. No NADPH formation was observed in the absence of substrates. Initial experiments indicated that the absorbance changed proportionally with

NADP in the presence of the complete incubation mixture up to an extinction of 0.340 to 0.400 units. Therefore, incubations were performed until this extinction was reached and then stopped.

2.1.6. Source of chemicals

All chemicals were obtained from Merck unless otherwise specified. GABA and 2,4-diaminobutyrate were obtained from Calbiochem; Triton X-100, 2-oxo-glutaric acid, disodium succinate, hyamine 10-X hydroxide, 2-mercaptoethanol were obtained from BDH Chemicals Ltd.; p-hydroxybenzaldehyde, 5-hydroxy-tryptophan, 5-hydroxytryptamine, noradrenaline, dopamine, adrenaline were obtained from FLUKA AG; $FeCl_3.6\ H_2O$, morphine.HCl, L-DOPA and strychnine-nitrate were obtained from Brocades, Haarlem; bicuculline, amino-oxy-acetic acid.HCl were obtained from K & K Laboratories; 3-mercaptopropionic acid, picrotoxine and thiosemicarbazide were obtained from Janssen Pharmaceutica; NAD, NADH, NADP and NADPH were obtained from Boehringen; GABASE was obtained from Worthington; (U-^{14}C)-GABA (spec. radioact. 4.6 mCi/mMol) and DL-1-^{14}C-glutamic acid (spec. radioact.: 29 mCi/mMol) were obtained from The Radiochemical Centre, Amersham; naphtalene, 2,5-diphenyl-oxazole, 1,4-bis-2-(5-phenyloxazolyl)-benzene and dioxane were obtained from Packard Instruments Company; 1-hydroxy-3-aminopyrrolidone-2 (HA-966) was a gift from AKZO, The Netherlands; di-n-propylacetate was a gift from LABAZ B.V., Maassluis, the Netherlands; naloxone was a gift from Endo Lab., Brussels-New York; DPI was a gift from Dr. A.R. Cools, Nijmegen; 4-N-hydroxy-2,4-diamino-butyrate was synthesized from HA-966 as described below; SSA was synthesized from monosodium glutamate as described by KAMMERAAT (1966).

4-N-hydroxy-2,4-diaminobutyrate was synthesized from HA-966 by refluxing for 3 h in boiling 12% HCl. The product was dried *in vacuo*, the residue was dissolved in a small volume of water and subsequently dried again *in vacuo*. The resulting oily solution was neutralized and the product was washed with ethanol and ether. NMR and infrared spectra were in accordance with its structure.

2.2. ASSAY AND SOME PROPERTIES OF GLUTAMATE DECARBOXYLASE

To obtain optimal incubation conditions the effect of incubation time, tissue concentration, substrate concentration and co-factor addition have been studied for glutamate decarboxylase using the radiochemical assay of ROBERTS & SIMONSEN (1963).

RESULTS

2.2.1. Time course of CO_2 production in the presence or absence of PLP

The time course of CO_2 production by glutamate decarboxylase was studied in the presence and absence of added PLP. The results in Fig. 2.2.1. demonstrate that CO_2 production was linear with incubation time. From these results an incubation time for routine measurement of glutamate decarboxylase activity of 60 min was selected.

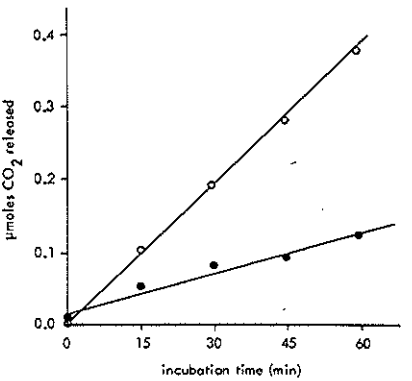


Fig. 2.2.1. The time course of CO_2 production by glutamate decarboxylase activity. The experiments were performed at 38°C in duplicate with (–o–o–) or without (–•–•–) added 0.4 mM PLP. For experimental conditions see 2.1.1.

2.2.2. Effect of tissue concentration

The effect of tissue concentration was studied under the standardized conditions in the presence of PLP. The results in Fig. 2.2.2. demonstrate that the formation of CO_2 is proportional with tissue concentration up to at least 12.5 mg tissue/ml.

2.2.3. Effect of glutamate concentration

The effect of substrate concentration was studied using from 5 to 25 mM glutamate in the presence of 0.4 mM PLP. The formation of CO_2 was estimated after 20 and 80 min of incubation. The results presented in Fig. 2.2.3. demonstrate that maximal glutamate decarboxylase activity was obtained with about 10 mM glutamate. No substantial increase in $^{14}CO_2$ production was observed with higher glutamate concentrations. The activities obtained after 20 min of incubation were about 15% higher when compared with the activities obtained

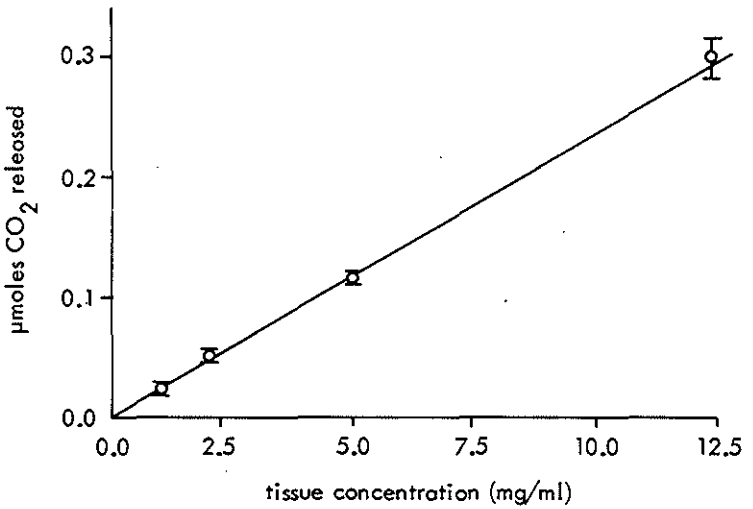


Fig. 2.2.2. The effect of tissue concentration on glutamate decarboxylase activity. The experiments were performed at 38°C with an incubation time of 60 min. For experimental conditions see 2.1.1.

with 80 min of incubation. If 0.05 mM AOAA was present during incubation instead of PLP, $^{14}CO_2$ production was similar to that observed in blanks incubated with boiled homogenate. In these blanks a small production of $^{14}CO_2$ occurred which was dependent on the concentration of the radioactive glutamate. The results in Fig. 2.2.4. demonstrate that CO_2 production calculated for blanks incubated with 10 mM glutamate is about 0.03 µmol/ml/h, which is about 7.8%of the production of CO_2 under standardized assay conditions with 9.0 mM glutamate.

DISCUSSION

A radiochemical method was used for the assay of glutamate decarboxylase activity in which CO_2 production was proportional to incubation time and tissue concentration. Recently, AOAA was shown to stimulate $^{14}CO_2$ release during the glutamate decarboxylase assay, which effect was ascribed to impurities present in commercially available radioactive glutamate (MILLER & MARTIN, 1973). However, when measured in the presence of Triton X-100 after extensive gassing with nitrogen, identical results are obtained when fluorimetrically

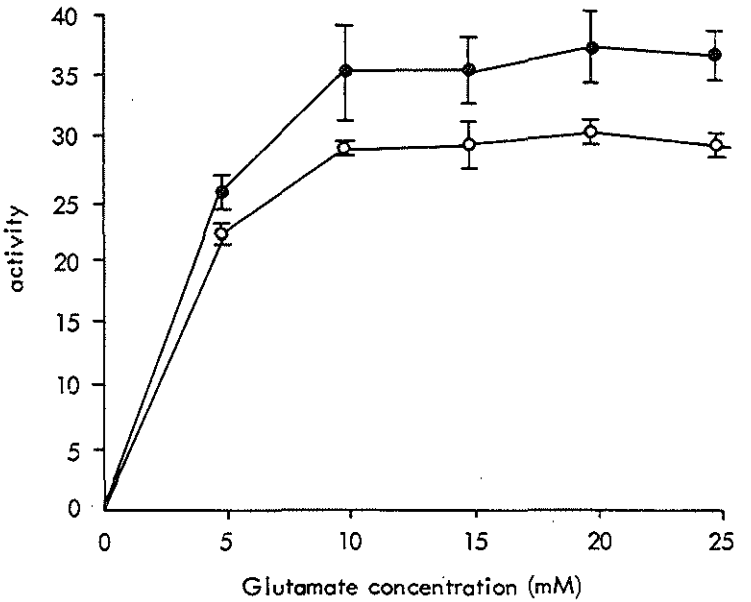


Fig. 2.2.3. The effect of glutamate on glutamate decarboxylase activity. Glutamate decarboxylase activity was estimated after 20 (–•–•–) or 80 (–○–○–) min of incubation. For experimental conditions see 2.1.1. Substrate solutions were prepared as follows: a solution containing 125 mM glutamate in buffer together with 2.5 μCi DL-1-^{14}C-glutamic acid (spec. act.: 29 mCi/mMol), was mixed with varying volumes of the buffer solution. In this way solutions of 25, 50, 75, 100 and 125 mM L-glutamate were obtained together with 0.5, 1.0, 1.5, 2.0 and 2.5 μCi/ml DL-1-^{14}C-glutamic acid, respectively. 0.2 ml of the substrate solutions was added to the incubation vessels giving final concentrations of 5, 10, 15, 20 and 25 mM L-glutamate in the incubation mixture. Activity is expressed as μmol/100 mg protein/h. Results are the mean ± S.E. of three experiments.

measured GABA formation is compared with radiochemically measured CO_2 release (MACDONNEL & GREENGARD, 1975; DRUMMOND & PHILLIPS, 1974). Since both GABA formation and CO_2 release are an estimate for glutamate decarboxylase activity, these results suggest that $^{14}CO_2$ release from radioactive impurities does not interfere with the assay of glutamate decarboxylase under these experimental conditions. Using similar incubation conditions the present experiments indicate that AOAA completely inhibited glutamate decarboxylase activity suggesting that no AOAA stimulated $^{14}CO_2$ release

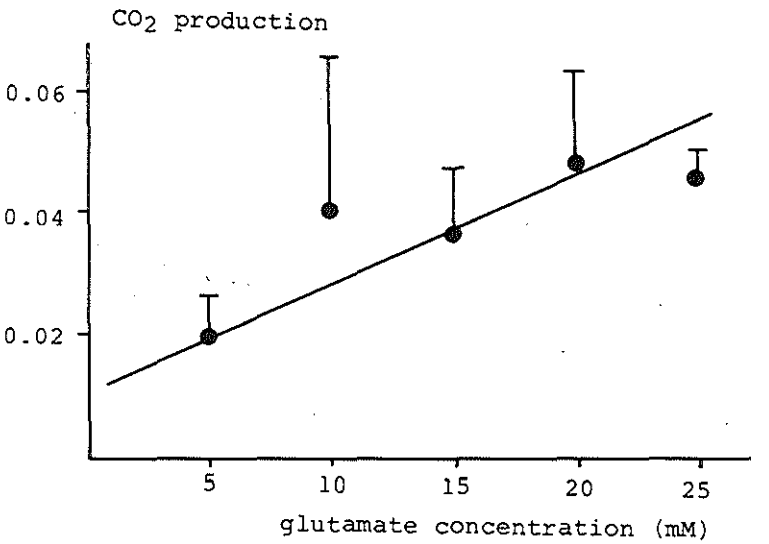


Fig. 2.2.4. The effect of substrate concentration on CO_2 production in blanks. The experimental conditions are similar to those described in the legend of Fig. 2.2.4, except that boiled homogenate was used instead of fresh tissue. The results are the mean ± S.E. of three experiments.

ocurred under the presently used incubation conditions. Using larger amounts of radioactive glutamate, however, some evidence was obtained for an initial burst of $^{14}CO_2$ formation, because the activity of glutamate decarboxylase as measured after 20 min of incubation was consistantly higher when compared with the activities measured after 80 min of incubation (Fig. 2.2.3.). This initial burst of $^{14}CO_2$ production probably remained undetected under standard assay conditions, because its contribution is only 2.5% of the total CO_2 liberated. A much larger deviation from linearity of the time course of

glutamate decarboxylase activity was reported to occur in the absence of added PLP (WOOD & PEESKER, 1972). The present results indicate that maximal glutamate decarboxylase activity can be obtained with about 10 mM glutamate. This value is in accordance with the reported K_m value for the isolated enzyme of 0.7 mM (WU et al., 1973). Using the same incubation conditions as described here, TAPPAZ et al. (1976) did not find any contribution from impurities during the assay of glutamate decarboxylase. A linear time course was found for at least one hour and a K_m for glutamate of 4 mM was reported. However, radiochemical glutamate decarboxylase assays using a high substrate concentration have been repeatedly reported (SIMS & PITTS, 1970; WOOD & PEESKER, 1972; BAYOUMI & SMITH, 1973; KIMURA & KURIYAMA, 1975). A high substrate concentration requires the use of increasing amounts of radioactive glutamate with the risk of an increasing contribution to glutamate decarboxylase activity of $^{14}CO_2$ produced from impurities. Therefore, the relative low affinity of glutamate for glutamate decarboxylase reported in some of these studies might be caused by the contribution of $^{14}CO_2$ release from impurities present in the radioactive glutamate.

2.3. MEASUREMENT OF GABA-TRANSAMINASE BY A COUPLED ENZYME METHOD

A variety of methods have been devised to measure GABA-transaminase activity (for review, see BAXTER, 1972). Most of these methods are not sensitive enough to permit measurements in small samples of brain. A sensitive fluorimetric method was described by PITTS et al. (1965) in which NADH formed during the conversion of SSA into succinate by means of purified kidney SSA-dehydrogenase was measured. A radiochemical method based upon the conversion of SSA by endogenous SSA-dehydrogenase was described by COLLINS (1972), while BUU & VAN GELDER (1974) used a coupled enzyme method for the assay of GABA-transaminase in subcellular fractions. The aim of this investigation was to combine these methods to avoid SSA-dehydrogenase isolation and purification and to permit measurements in small tissue samples. Since the properties of SSA-dehydrogenase are of importance for the GABA-transaminase assay described we have also investigated the kinetic parameters of this dehydrogenase.

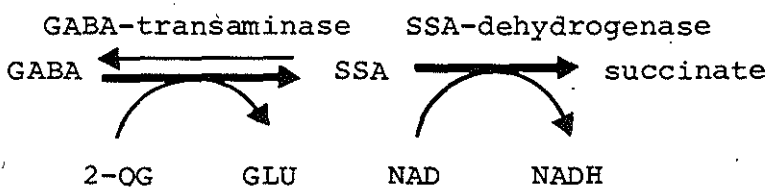


Fig. 2.3.1. The coupled GABA-transaminase assay using NADH formation as an estimate of GABA-transaminase activity.

2.3.1. Characteristics of GABA-transaminase

Buffers and salt addition. Transamination of GABA by the action of GABA-transaminase results in the formation of SSA which can be oxidized by SSA-dehydrogenase in the presence of the cofactor NAD (Fig. 2.3.1). If GABA-transaminase is rate limiting and if no substantial accumulation of SSA occurs, NADH formation can be used as an estimate for GABA-transaminase activity. Initial measurements of GABA-transaminase activity using glycylglycine-KOH as a buffer resulted in a low rate of NADH formation which could be considerably increased by addition of salt or Tris-buffer. A survey of the results obtained with different buffers as well as the effect of salt addition is given in Table 2.3.1a. SSA-dehydrogenase was also inhibited in glycylglycine-KOH buffer

Table 2.3.1.

Effect of buffer and salt addition on GABA-transaminase and SSA-dehydrogenase activity

	BUFFER	ACTIVITY	(N)	%	P
(a)	GABA-transaminase activity				
	30 mM glycylglycine-KOH	6.1; 6.4		100	–
	30 mM glycylglycine-KOH + 200 mM salt*	12.4; 12.6		195	–
	50 mM Tris-HCl + 30 mM glycylglycine	27.2 ± 1.5	(3)	100	–
	50 mM Tris-HCl + 30 mM glycylglycine + 200 mM salt*	13.6 ± 0.9	(3)	50	0.001
	125 mM Tris-HCl + 30 mM glycylglycine	24.2 ± 1.7	(3)	100	–
	125 mM Tris-HCl + 30 mM glycylglycine + 200 mM salt*	12.2 ± 0.1	(3)	50	0.005
	50 mM Tris-HCl	40.3 ± 1.4	(4)	100	–
	50 mM Tris-HCl + 200 mM NaCl	18.4 ± 1.1	(4)	46	0.001
	50 mM Tris-HCl + 200 mM KCl	18.2 ± 1.0	(4)	45	0.001
	50 mM Tris-HCl + 200 mM choline chloride	18.3 ± 0.9	(4)	46	0.001
	50 mM Tris-HCl + 200 mM sodium isethionate	21.2 ± 1.1	(3)	53	0.001
(b)	SSA-dehydrogenase activity				
	30 mM glycylglycine-KOH	1.1 ± 0.5	(3)	–	–
	50 mM Tris-HCl + 30 mM glycylglycine	7.1 ± 3.8	(3)	–	–
	50 mM Tris-HCl	54.5 ± 2.6	(3)	–	–

(a) The incubation mixture contained: 3.0 mM GABA; 2.0 mM 2-oxoglutaric acid; 20 mM 2-mercaptoethanol; 1.1 mM NAD and 6.7 mg tissue/ml. Generally, GABA-transaminase activity was calculated on the base of time courses incubated between 0 and 4 h. Incubations were performed in the presence or absence of salt as indicated. In glycylglycine-KOH buffer only measurements with KCl and NaCl were performed. For the buffer mixture 50 mM Tris-HCl + 30 mM glycylglycine without salt, activity was calculated from fluorescence production after one hour of incubation, since the time course sharply flattened between 1 and 4 h. Results are the mean ± S.E. with the number of experiments given in parenthesis. Percentages and P-values refer to the effect of salt in comparison to appropriate controls. P-values were estimated using Student's t-test.

(b) Since SSA-dehydrogenase activity was measured using GABA-transaminase assay conditions the incubation mixture was the same as described under (a) with GABA being replaced by 0.3 mM SSA. SSA-dehydrogenase activity was calculated from time courses with an incubation time between 0 and 60 min.

* Mean ± S.E. of separate measurements with NaCl and KCl for glycylglycine-buffer and with NaCl, KCl, choline chloride and sodium isethionate in other buffer mixtures.

(Table 2.3.1b) suggesting that SSA-dehydrogenase was probably rate limiting during the assay of GABA-transaminase in the presence of this buffer. The

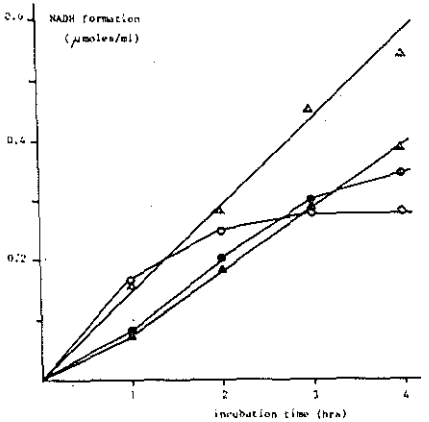


Fig. 2.3.2. The time course of GABA-transaminase activity in the presence of Tris-glycyl-glycine-KOH buffers and the effect of salt addition. The reaction mixture contained: 50 mM Tris + 30 mM glycylglycine-KOH buffer without (–○–○–) or with (–●–●–) 200 mM KCl or 125 mM Tris-glycylglycine buffer without (–△–△–) or with (–▲–▲–) 200 mM KCl; 3.0 mM GABA; 2.0 mM 2-oxoglutaric acid; 20 mM 2-mercaptoethanol; 1.1 mM NAD and 6.7 mg tissue/ml (0.6 ml Triton-treated homogenate) in total volume of 2.25 ml.

experiments shown in Fig. 2.3.2 demonstrate that in the presence of a mixture of 50 mM Tris and 30 mM glycylglycine-KOH non-linear curves were obtained with an almost complete inhibition of NADH formation after 2 h of incubation. Salt addition resulted in an inhibition of the initial rate of NADH formation, while non-linearity was no longer observed. Therefore it was decided to measure GABA-transaminase activity using Tris-HCl as a buffer. Under these conditions NADH formation was linear with incubation time for 3 h in the presence as well as in the absence of added salts, while it was completely inhibited by 0.045 mM AOAA as shown in Fig. 2.3.2. Addition of NaCl, KCl, choline chloride or sodium isethionate resulted in an inhibition of NADH formation to the same extent (about 50%), suggesting that this inhibition was caused by ionic strength. This was further confirmed by studying the effect of both NaCl and sodium isethionate on GABA-transaminase activity. By plotting log (activity) against the square root of ionic strength (Debye-Hückel limiting law) a straight line was found with a slope of −1.08 (Fig. 2.3.4.) indicating that the inhibition was caused by the increase in ionic strength (DEBYE & HÜCKEL, 1923).

Effect of temperature. The activity of GABA-transaminase was estimated at 22°C and 37°C in the presence of NaCl, KCl, choline chloride or sodium isethionate ($HOCH_2CH_2SO_3$-Na). These activities were 18.6 ± 0.1 at 22°C and

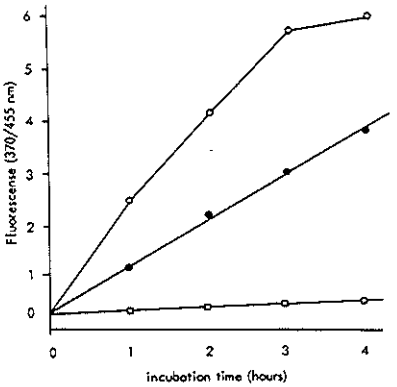


Fig. 2.3.3. The time course of GABA-transaminase activity in Tris-HCl. The reaction mixture contained: 50 mM Tris-HCl, pH 8.5; 3.0 mM GABA; 2.0 mM 2-oxoglutaric acid; 1.1 mM NAD; 20 mM 2-mercaptoethanol and 6.7 mg tissue/ml in the absence of salts (–○–○–), in the presence of NaCl, KCl, choline chloride or sodium isethionate (all 200 mM) (–●–●–) or in the presence of 0.045 mM AOAA (–□–□–). The reaction was started by addition of tissue.

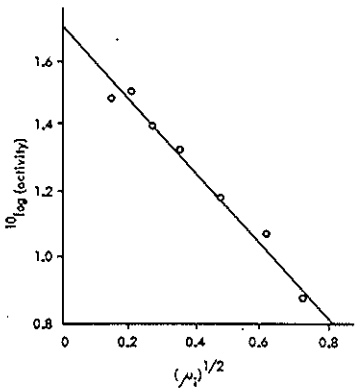


Fig. 2.3.4. Effect of increase in ionic strength (μ_i) on GABA-transaminase activity. For incubation conditions see the legend of Fig. 2.3.3.

47 ± 1.0 μmol/g tissue/h at 37°C, respectively, indicating a ratio of 2.53 for GABA-transaminase activities at these temperatures.

Tissue concentration. The effect of tissue concentration on GABA-transaminase activity was measured in the presence of 1.1 or 0.34 mM NAD (Fig. 2.3.5.). In both cases fluorescence production was proportional to tissue concentration up

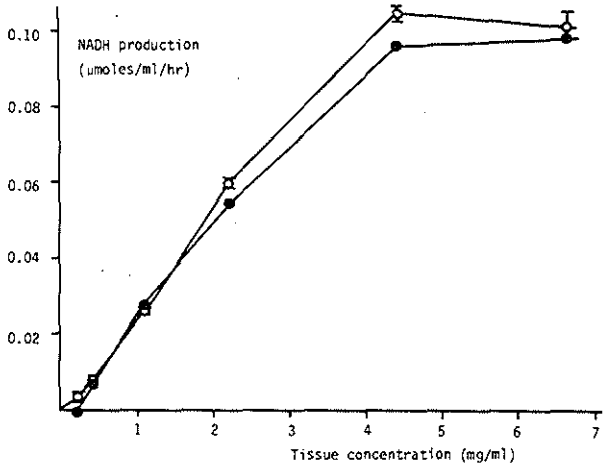


Fig. 2.3.5. Effect of tissue concentration on GABA-transaminase activity. The incubation mixture contained: 50 mM Tris-HCl, pH 8.5; 3.0 mM GABA; 2.0 mM 2-oxoglutaric acid; 20 mM 2-mercaptoethanol; 0.34 (–o–o–) or 1.1 (–•–•–) mM NAD; varying amounts of tissue. With 1.1 mM NAD one homogenate was used, while the results with 0.34 mM NAD represent the mean + S.E. for 3 experiments.

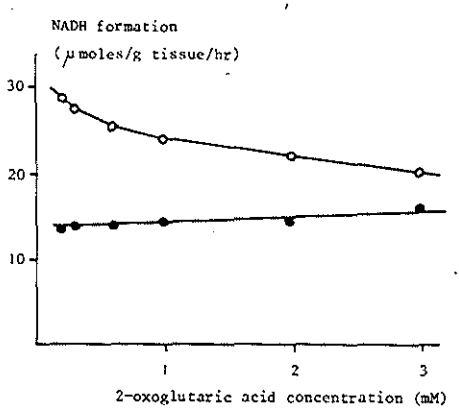


Fig. 2.3.6. The effect of 2-oxoglutaric acid on GABA-transaminase activity. The incubation mixture contained: 50 mM Tris-HCl, pH 8.5; 3.0 mM GABA; 20 mM 2-mercaptoethanol; 1.1 mM NAD; 2.2 mg tissue/ml; varying concentrations of 2-oxoglutaric acid and no NaCl (–o–o–) or 200 mM NaCl (–•–•–).

to about 4.44 mg tissue/ml. Higher tissue concentrations did not cause a substantial increase in fluorescence. Therefore, a tissue concentration of 2.22 mg/ml was selected for routine measurements of GABA-transaminase activity.

2-oxoglutaric acid and NaCl. The effect of varying concentrations of 2-oxoglutaric acid was studied in the presence or absence of 200 mM NaCl. The results in Fig. 2.3.6. demonstrate that GABA-transaminase is maximally active at the lowest concentration of 2-oxoglutaric acid studied, i.e. 0.2 mM. Higher concentrations were found to be inhibitory, indicating that the affinity of 2-oxoglutaric acid for GABA-transaminase is relatively high with a K_m lower than 0.2 mM. Addition of 200 mM NaCl caused an inhibition of 20% at 3 mM and 45% at 0.2 mM 2-oxoglutaric acid, while no inhibition by 2-oxoglutaric acid was observed. By plotting the reciprocal of 2-oxoglutaric acid concentration against activity, a straight line was obtained from which a K_i for 2-oxoglutaric acid in the absence of 200 mM NaCl of 7.2 mM was obtained.

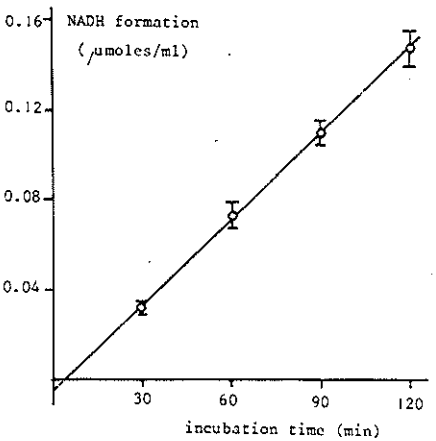


Fig. 2.3.7. Estimation of SSA accumulation during GABA-transaminase assay. The incubation mixture contained: 50 mM Tris-HCl, pH 8.5; 3.0 mM GABA; 2.0 mM 2-oxoglutaric acid; 20 mM 2-mercaptoethanol; 0.9 mM NAD and 2.22 mg tissue/ml. Extrapolation to zero time was averaged for three experiments resulting in an estimation of 0.0046 ± 0.0016 mM accumulated SSA during the assay.

SSA accumulation during GABA-transaminase measurement. To study SSA accumulation during the GABA-transaminase assay time courses of NADH production with 2.22 mg/ml tissue concentration were extrapolated to zero time for individual experiments to see if a lag-time period was observed (Fig. 2.3.7.). The amount of accumulated SSA that can be calculated from the difference between the extrapolated zero-time fluorescence and the actually measured fluorescence at zero time, was found to be 0.0046 ± 0.0016 mM (mean ± S.E., n = 3). This should correspond to the steady-state concentration of SSA during the GABA-transaminase assay.

GABA-transaminase assay by different methods. In addition to the method described above, the radiochemical method of COLLINS (1972) was used in which GABA-transaminase activity is measured as the amount of (^{14}C)-GABA converted into (^{14}C)-SSA + (^{14}C)-succinate. With this method the acidic products are separated from GABA and measured together in one fraction. Experiments using the standardized fluorimetric assay were run in parallel for reasons of comparison. The results in Table 2.3.2. show that with both methods similar activities were obtained. Also, 200 mM NaCl was inhibitory in both systems, though the extent of inhibition was not the same (Table 2.3.2.).

Table 2.3.2.

Comparison of the fluorimetric and the radiochemical GABA-transaminase assay with and without 200 mM NaCl

Addition	GABA-transaminase activity (μmol/g/h)	
	radiochemical method	fluorimetric method
none	28.1	30.7
200 mM NaCl	17.3	11.8

The experiments were performed exactly as described in the legend of Table 2.3.3. The tissue concentration was 6.7 mg tissue/ml. For both methods blanks were used containing the complete incubation mixture together with 0.045 mM AOAA.

Individual values obtained from experiments with homogenates from total brain or different brain regions, using the radiochemical and the fluorescent method in parallel, were nearly identical (Table 2.3.3.). This indicates that during the GABA-transaminase assay SSA is completely converted into succinate. However, for the kidney the results obtained with both methods were not correlated. Therefore, in brain tissue the measurement of GABA-transaminase via the conversion of SSA into succinate does not seem to introduce any errors when using NADH formation as an estimate of GABA-transaminase activity.

2.3.2. Characteristics of SSA-dehydrogenase

The properties of SSA-dehydrogenase are of particular importance for a valid assay of GABA-transaminase activity. SSA must have a high affinity for the dehydrogenase and SSA-dehydrogenase activity must exceed that of GABA-transaminase sufficiently to prevent substantial accumulation of the intermediate SSA. Such an accumulation of intermediate SSA may cause erroneous results for

Table 2.3.3.

Comparison of the fluorimetric and radiochemical assay of GABA-transaminase in different brain regions and tissues

Tissue	GABA-transaminase activity (μmol/g/h)			SSA-dehydrogenase activity (μmol/g/h)	SSA-dehydrogenase[3] / GABA-transaminase
	from time course[1]	radiochemical method	fluorescent[2] method		
Total brain	34.6 ± 1.7 (3)	31.3; 37.7	31.4; 37.7	63.6 ± 3.6 (3)	1.84 ± 0.05 (3)
striatum	–	23.6 ± 1.0 (3)	27.3 ± 1.4 (3)	77.1 ± 3.3 (3)	2.83 ± 0.08 (3)
midbrain	–	26.9 ± 2.3 (3)	29.6 ± 1.9 (3)	92.2 ± 0.8 (3)	3.14 ± 0.17 (3)
cerebellum	–	25.5 ± 2.1 (3)	27.9 ± 1.4 (3)	76.8 ± 0.9 (3)	2.77 ± 0.13 (3)
kidney	–	13.5 ± 1.2 (3)	7.6 ± 1.4 (3)	13.7 ± 0.7 (3)	1.03 ± 0.04 (3)

GABA-transaminase activity was measured in a medium containing: 50 mM Tris-HCl, pH 8.5; 3.0 mM GABA; 2.0 mM 2-oxoglutaric acid; 20 mM 2-mercaptoethanol; 0.9 mM NAD and 2.22 mg tissue/ml. NADH formation was measured after 120 min for brain regions and kidney, while for total brain NADH formation was measured after 0, 30, 60, 90 and 120 min of incubation. For the radiochemical method the incubation mixture contained 1.0 mM succinate and 0.9 μCi/ml of U-^{14}C-GABA (spec. act.: 4.6 mCi/mMol) in addition to the incubation mixture described above, while the incubation time was 120 min. Blanks for the radiochemical method contained 0.045 mM AOAA in addition to the complete incubation mixture. For the measurement of SSA-dehydrogenase activity under GABA-transaminase assay conditions, the incubation mixture was similar to that described for the fluorimetric GABA-transaminase assay, except that 0.3 mM SSA was present instead of GABA. Results are the mean ± S.E. with the number of experiments in parenthesis.

[1] Results are based on the slope of the time course of NADH formation.

[2] Results are based on the measurement of NADH formation after 120 min of incubation.

[3] For the ratio of SSA-dehydrogenase and GABA-transaminase activity the results with the fluorimetric method were compared, except for the kidney where for GABA-transaminase activity the results with the radiochemical method were used.

the GABA-transaminase activity. Therefore, the properties of SSA-dehydrogenase were studied to see whether these two conditions were fulfilled.

SSA-concentration. To estimate SSA concentration enzymatically the time course of NADH formation was measured until no further increase in NADH concentration could be observed. SSA concentration was calculated from maximal fluorescence production using spectrophotometrically standardized NADH. Maximal fluorescence production was proportional with the amount of added SSA (Fig. 2.3.8.).

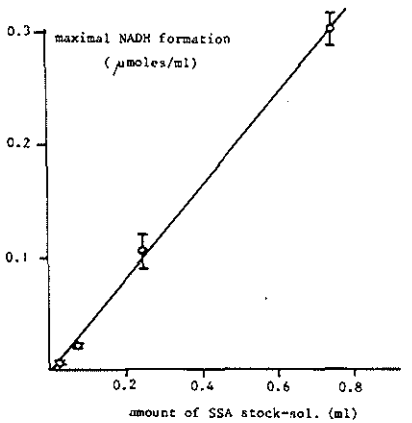


Fig. 2.3.8. Estimation of SSA concentration using rat brain SSA-dehydrogenase. A concentrated SSA solution was diluted with 50 mM Tris-solution and adjusted to pH 8.5 with KOH. The incubation mixture contained: 50 mM Tris-HCl, pH 8.5; 4.44 mg tissue/ml; 0.46 mM NAD; 10 mM 2-mercaptoethanol and substrate in a total volume of 2.25 ml. Maximal fluorescense was estimated by following the time course of NADH formation from 0 to 180 or 240 min. The results are the mean ± S.E. of three experiments.

From the time courses mentioned above it is possible to obtain values for the K_m and V_m using the integrated Henri-Michaelis-Menten procedure (SEGEL, 1975). A K_m of 138 ± 15 μM and a V_m of 64.5 ± 7.0 μmol/g/h (mean ± S.E., n = 3) were obtained using a tissue concentration of 4.44 mg/ml. Although the V_m was sufficiently high to allow the use of SSA-dehydrogenase for the coupled assay of GABA-transaminase, the affinity was particularly low. These data were in conflict with studies demonstrating a high affinity of SSA for the dehydrogenase with a K_m of about 2-4 μM (EMBREE & ALBERS, 1964; KAMMERAAT & VELDSTRA, 1968). They were also in variance with the results obtained for the GABA-transaminase assay described in the previous paragraph, suggesting that no accumulation of SSA occurred. This paradoxial finding prompted us to

study the kinetic properties of SSA-dehydrogenase in more detail. Because we used a rather high tissue concentration (4.44 mg/ml) as compared to KAMMERAAT & VELDSTRA (1968), using 0.33 mg/ml, we have investigated the effect of tissue concentration. The effect of NaCl was also studied for reasons of comparison with the GABA-transaminase results and because NaCl might change the properties of the aggregated SSA-dehydrogenase as was found for *pseudomonas* SSA-dehydrogenase (CALLEWAERT et al., 1973; ROSEMBLATT et al., 1973).

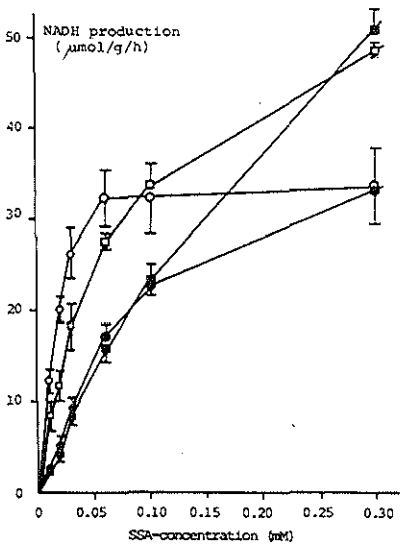


Fig. 2.3.9. Effect of tissue concentration and salt addition on kinetic parameters of SSA-dehydrogenase; saturation curves. The incubation mixture contained: 50 mM Tris-HCl, pH 8.5; 8.2 mM 2-mercapthanol; 0.23 mM NAD and 0.01 to 0.3 mM SSA. The incubation time was 10 min for the experiments at 4.44 and 60 min for the experiments at 0.44 mg tissue/ml. The experimental points are: without NaCl at 0.44 (–○–○–) and 4.44 (–●–●–) mg tissue/ml and with 200 mM NaCl at 0.44 (–□–□–) and 4.44 (–■–■–) mg tissue/ml. The results are the mean ± S.E. for three experiments. Activity is expressed as µmol/g/h.

Effect of tissue concentration and NaCl addition. Incubations were performed with 0.44 and 4.44 mg tissue/ml in the presence or absence of 200 mM NaCl and the resulting saturation curves are presented in Fig. 2.3.9. The corresponding integrated Henry-Michaelis-Menten curves are shown in Fig. 2.3.10. and the calculated kinetic constants are summarized in Table 2.3.4.
The integrated Henri-Michaelis-Menten curve had to be used because a substan-

tial fraction of SSA was converted into succinate during the course of the reaction. Therefore, the usual linear plots based on the Henri-Michelis-Menten equation (e.g., 1/v versus 1/S) could not be used to calculate K_m and V_m. Since the integrated Henri-Michelis-Menten curve is valid over the entire course of the reaction, points can be used for the calculation of kinetic parameters with between 10 and 90% conversion of substrate. The simplest integrated rate equation is:

$$V_m\, t = 2.3\, K_m \log\frac{s_o}{s} + (s_o - s).$$

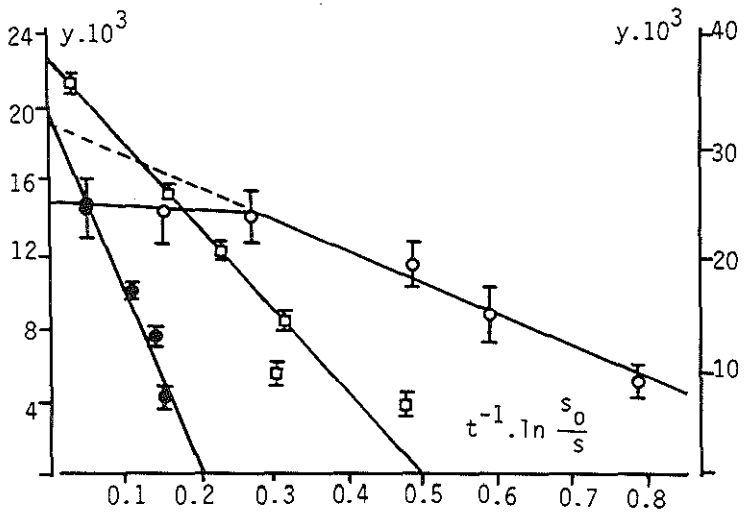


Fig. 2.3.10 Corresponding integrated Henri-Michaelis-Menten curves of three of the saturation curves given in Fig. 2.3.9. A linear transformation of the integrated Henri-Michaelis-Menten equation was used which is analogue to the plot of v versus v/s (the Woolf-Augustinsson-Hofstee plot):

$y = -K_m.t^{-1}.\ln\frac{s_o}{s} + V_m$. Here is y equal to the amount of product formed during the incubation time t, s_o is the initial substrate concentration and s is the substrate concentration at the end of the incubation period. To estimate kinetic parameters of SSA-dehydrogenase with different NaCl or tissue concentration y was plotted versus $t^{-1}.\ln\frac{s_o}{s}$. The slope of the obtained straight line is equal -Km (dimension: mM) while the intercept with the vertical axis is equal to V_m (dimension: µmol/ml formed). To demonstrate the effect of tissue concentration in one figure it was necessary to introduce a second vertical scale because of differences in tissue concentration and incubation time. The vertical scale on the right refers to the results with 4.44 mg tissue/ml and is extended 1.67 times in comparison with the vertical scale on the left refering to the experiments with 0.44 mg tissue/ml. As a result different integrated Henri-Michaelis-Menten curves exhibiting the same V_m will intercept the vertical axis at the same place.

which can be rearranged to:

$$y\,t^{-1} = -K_m\,(2.3\,t^{-1}\log\frac{s_o}{s_o - y}) + V_m$$

A more extensive study of the integrated Henri-Michaelis-Menten equation is described by SEGEL (1975). The activity of the dehydrogenase, expressed as μmol/ml formed during incubation, is plotted against $\ln(\frac{s_o}{s_o - y})$. In this formula s_o is equal to the initial substrate concentration, while y is equal to the amount of product formed. The slope of the resulting straight line is equal $-K_m$, while V_m can be calculated from the intercept with the vertical axis. At a concentration of 0.44 mg tissue/ml a biphasic curve was found in the absence of NaCl. The calculation of K_m and V_m values was based upon measurements of SSA-dehydrogenase activity using substrate concentrations between 0.01 and 0.06 mM SSA. No increase in NADH production was found between 0.06 and 0.3 mM SSA. Increase of tissue concentration resulted in a decrease of affinity as indicated by the sharp increase of the slope of the integrated Henri-Michaelis-Menten curve (Fig. 2.3.10). NaCl addition at a concentration of 0.44 mg/ml tissue decreased affinity and resulted in a straight integrated Henri-Michaelis-Menten curve. At 0.3 mM SSA the addition of NaCl increased SSA-dehydrogenase activity considerably. This activation by NaCl, which can be expressed as the ratio of V_m in the presence of NaCl and maximal activity in the absence of NaCl, as measured between 0.06 and 0.3 mM SSA, was 1.76 ± 0.07 (mean + S.E., n = 3). Despite this activation by NaCl at higher substrate concentration no effect was found on V_m, as calculated from the linear part of the integrated Henri-Michaelis-Menten curve. At a concentration of 4.44 mg tissue/ ml both K_m and V_m were changed significantly by NaCl (Table 2.3.4).

Table 2.3.4.

Effect of tissue concentration and salt addition on kinetic parameters of SSA-dehydrogenase

Tissue concentration (mg/ml wet wt)	K_m (μM)		V_m (μmol/g/h)	
	control	200 mM NaCl	control	200 mM NaCl
0.44	19.3 ± 7.0	59.7 ± 16.5[1]	45.4 ± 7.9	55.1 ± 5.7
4.44	90.7 ± 11.5[3]	320.4 ± 94.5[1,4]	44.0 ± 5.7	103.8 ± 14.8[2,5]

Kinetic parameters were calculated using the integrated Henry-Michaelis-Menten equation. For the calculations at high tissue concentration measurements with 20 and 10 μM were omitted because activity was too low in comparison with V_m. Kinetic parameters calculated from three separate experiments were averaged, the mean ± S.E. being indicated. For further details see the legend of Fig. 2.3.10.

Statistics: The significance of the results was determined using Student's t-test.

Effect of salt addition in comparison with controls without salt: [1] $p < 0.05$; [2] $p < 0.025$.

Effect of increase in tissue concentration: [3] $p < 0.005$; [4] $p < 0.025$; [5] $p < 0.05$.

NAD-concentration. The results in Fig. 2.3.11. demonstrate that optimal NADH formation is obtained with about 0.3 mM NAD. Concentrations of NAD as low as possible have to be used because the concentration of NAD is directly related to the blank fluorescence (about 2% of NAD is converted into a fluorophore with similar properties as the fluorophore formed from NADH). An improvement of the GABA-transaminase and the SSA-dehydrogenase assay might be to decrease the concentration of NAD. The decrease of NAD to about 0.3 mM did not affect the GABA-transaminase reaction as indicated by the results in Fig. 2.3.5.

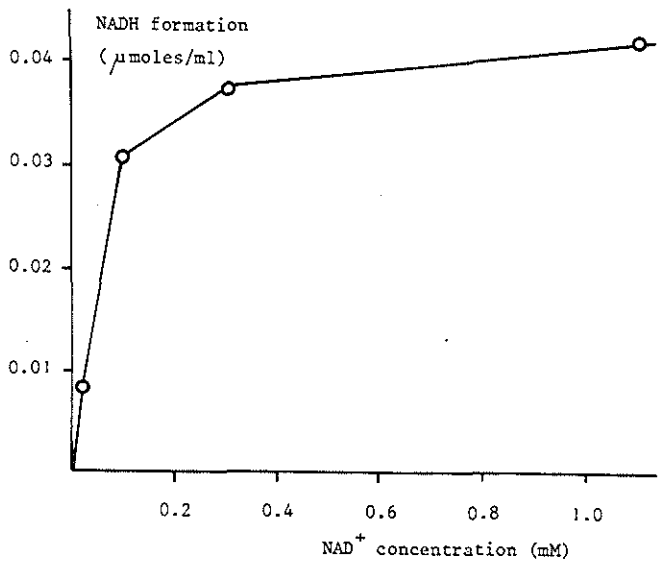


Fig. 2.3.11. The effect of NAD on SSA-dehydrogenase activity. The incubation mixture contained: 50 mM Tris-HCl, pH 8.5; 10 mM 2-mercaptoethanol; 0.3 mM SSA; 0.44 mg tissue/ml and varying amounts of NAD. The incubation time was 2 h.

SSA-dehydrogenase using GABA-transaminase assay conditions. Since the properties of SSA-dehydrogenase can vary considerably with the incubation conditions used, it is of importance to study the kinetic properties of the enzyme under conditions identical to those used for the GABA-transaminase assay. Therefore, two incubation systems were used: in one system GABA was replaced by SSA, while in the second system SSA-dehydrogenase was measured in the presence of GABA, while AOAA was added to the incubation mixture to prevent formation of SSA from GABA. The time course with tissue concentrations of 2.22 and 6.67 mg tissue/ml using the replacement of GABA by SSA is

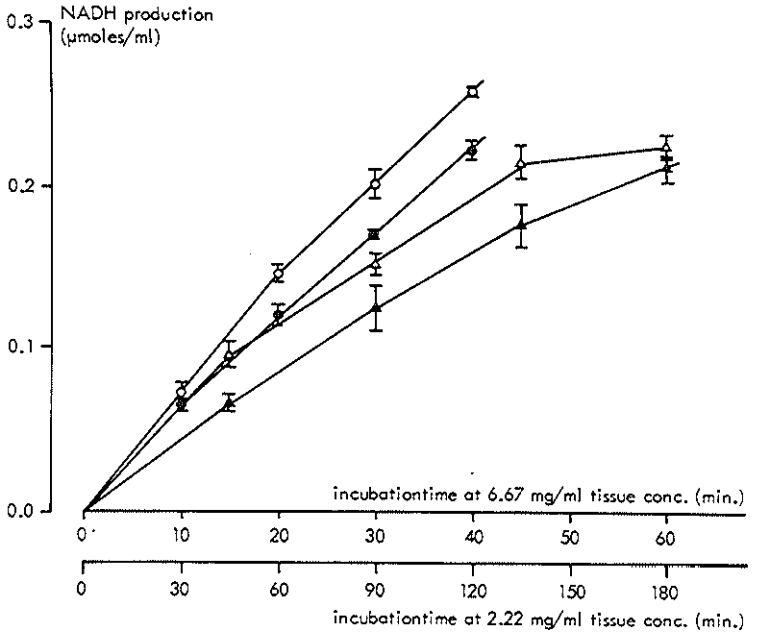


Fig. 2.3.12. The time course of NADH production by SSA-dehydrogenase; effect of tissue concentration and salt addition. The incubation mixture contained: 50 mM Tris-HCl, pH 8.5; 2.0 mM 2-oxoglutaric acid; 20 mM 2-mercaptoethanol; 0.3 mM SSA and brain tissue. The time scale at the higher tissue concentration is extended three times in comparison with that at lower tissue concentration to compensate for the three times higher potential rate of NADH formation at a concentration of 6.67 mg tissue/ml in comparison with 2.22 mg/ml. Experimental points (mean ± S.E. of three experiments) refer to: 2.22 mg tissue/ml without (–○–○–) and with (–●–●–) 200 mM NaCl, 6.67 mg tissue/ml: without (–△–△–) and with (–▲–▲–) 200 mM NaCl.

shown in Fig. 2.3.12. Addition of 200 mM NaCl was slightly inhibitory at both tissue concentrations. At the lower tissue concentration NADH production appeared to be proportional with incubation time in the presence as well as in the absence of NaCl. Increasing the tissue concentration, however, resulted in a non-linear time course with a decreasing rate of NADH formation during the course of the reaction. This effect could be demonstrated both in the presence and in the absence of 200 mM NaCl. In the second incubation system similar time courses were obtained showing that the conversion of SSA into succinate was not affected by the presence of GABA and AOAA.

The effect of different tissue concentrations on NADH production showed that

SSA-dehydrogenase activity was proportional with tissue concentrations up to 4.44 mg tissue/ml. Increase of tissue concentration to 6.67 mg tissue/ml did not increase NADH production proportionally.

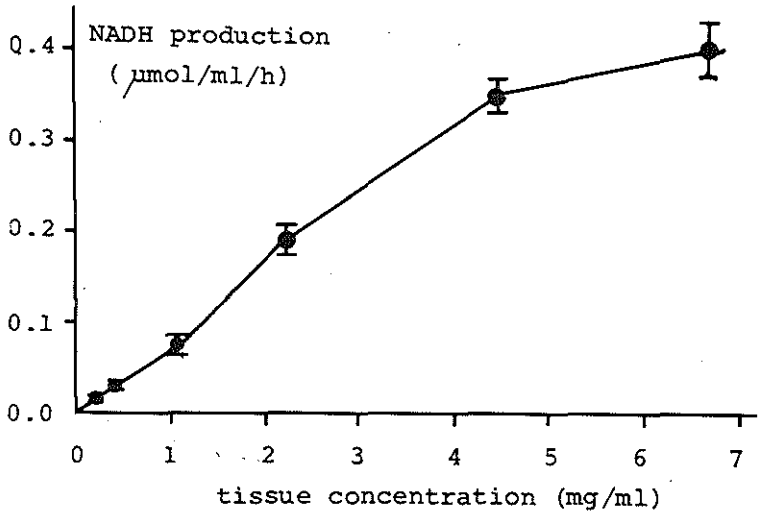


Fig. 2.3.13. The effect of tissue concentration on SSA-dehydrogenase activity. The incubation mixture was similar as described in the legend of Fig. 2.3.12. Solutions with 0.22 to 1.11 mg tissue/ml were incubated for 60 min, while the remaining solutions were incubated for 30 min. Results are the mean ± S.E. of four experiments.

2.3.3. Discussion

A high as well as a low GABA-transaminase activity have been reported with a glycylglycine-KOH buffer using the same radiochemical procedure (COLLINS, 1972; BEART & JOHNSTON, 1972). In the present study low activities for GABA-transaminase and SSA-dehydrogenase were found in this buffer, while both activities could be increased considerably by addition of Tris-HCl. These results suggest that the decrease in SSA-dehydrogenase activity is responsible for the decreased GABA-transaminase activity since the latter enzyme was assayed via SSA-dehydrogenase. Therefore it is concluded that the use of a glycylglycine-KOH buffer for GABA-transaminase assays using the coupled enzyme method should be avoided. The effect of salt addition on SSA-dehydrogenase activity will be discussed further in chapter 2.4.

If GABA-transaminase was measured in Tris-HCl buffer addition of salt was

found to inhibit NADH production. The effect of salt obeyed the Debye-Hückel limiting law indicating that this inhibition of GABA-transaminase was probably caused by increased ionic strength. Since the slope of the straight line is negative, it can be concluded that solute ions interacting with charged groups on the enzyme surface have an opposite charge (DEBYE & HÜCKEL, 1923). Since GABA-transaminase activity was measured via SSA-dehydrogenase the possibility of an inhibition of the dehydrogenase by salt must be considered. NaCl addition decreased the affinity of SSA for the dehydrogenase (Table 2.3.4) and may increase the concentration of intermediate SSA during the GABA-transaminase assay. This will result in a prolonged lag-time period, while it does not effect the rate of NADH formation. The experiments with glycyl-glycine-KOH buffer clearly show that inhibition of SSA-dehydrogenase might result in an apparent inhibition of GABA-transaminase. The experiments with the radiochemical method showed that salt inhibition was also observable under conditions where SSA accumulation does not cause an underestimation of GABA-transaminase activity. The possibility of increased GABA formation via the backward reaction, however, is still valid. Therefore, the observed inhibition of GABA-transaminase by salt can either result from an increased rate of GABA formation from accumulated SSA via the backward reaction or from a direct effect of salt on GABA-transaminase.

The experiments with varying concentrations of 2-oxoglutaric acid support the latter possibility. Because 2-oxoglutaric acid has no effect on brain SSA-dehydrogenase, this inhibition of GABA-transaminase by high concentrations of 2-oxoglutaric acid represents a direct effect on the transaminase. GABA-transaminase is active via a so-called Bi-Bi-Random mechanism (JOHN & FOWLER, 1976), 2-oxoglutaric acid is probably compatitive with GABA as observed also by MAITRE et al. (1975). This Bi-Bi-Random mechanisms means that GABA is firstly transaminated into SSA, while the amino-group remains bound to the enzyme. Thereafter, 2-oxoglutaric acid reacts with the enzyme and is converted into glutamate. Since no inhibition by 2-oxoglutaric acid can be observed in the presence of 200 mM NaCl, both are probably competitive with GABA for its binding sites. The effect of NaCl on GABA-transaminase may thus represent a mixture of competitive antagonism of NaCl with both GABA and 2-oxoglutaric acid for the same active site. This suggests that the inhibition of GABA-transaminase by NaCl, KCl, choline chloride and sodium isethionate is caused by a direct effect on this enzyme. The salt inhibition derived from the use of incubation mixtures with different ionic strengths might partly explain the large variation in GABA-transaminase reported in the literature (SALGANICOF & DE ROBERTIS, 1965; VAN KEMPEN et al., 1965; PITTS et al., 1965; COLLINS, 1972; BEART & JOHNSTON, 1972; WOOD & PEESKER, 1973).

It has been reported that a partially purified mouse brain GABA-transaminase has a strong tendency to aggregate, as indicated by a sharp decrease in activity as

a function of enzyme concentration (WAKSMAN & ROBERTS, 1965). The same phenomenon of enzyme aggregation might be responsible for the decreased specific activity of GABA-transaminase at tissue concentrations above 4.44 mg/ml reported here (Fig. 2.3.5). However, SSA-dehydrogenase might also contribute to the observed decrease in activity with tissue concentration since the affinity of SSA for the dehydrogenase is relatively low at high tissue concentrations. Because aggregation was not observed with a more extensively purified preparation (SCHOUSBOE et al., 1973) the occurrance of enzyme aggregation might depend on the extent of purification of the enzyme.

According to SEGEL (1975) the following conditions are necessary for a valid coupled assay for GABA-transaminase. Firstly, the primary reaction must be zero order with respect to substrate over the assay time and it must be irreversible. The first condition is met since only a small fraction of GABA or 2-oxoglutaric acid is utilized during the chosen assay period of 2 h, viz. 4.9 and 7.4% respectively at a concentration of 2.22 mg tissue/ml. Irreversibility is assumed because of the continuous removal of SSA in the second stage reaction. Secondly, the dehydrogenase reaction must be first order in respect to its substrate and must also be irreversible. This condition is met if SSA-dehydrogenase is sufficiently in excess over GABA-transaminase to ensure that the concentration of intermediate SSA will be small in comparison to its K_m for the dehydrogenase. Because accurate and direct measurements of SSA concentration during GABA-transaminase assay are lacking, only indirect evidence has been presented to show that the steady state concentration of SSA is sufficiently low to permit the use of the coupled assay for the estimation of GABA-transaminase activity. Clearly, accumulation of SSA to some extent is needed to activate the dehydrogenase sufficiently. The amount of SSA accumulated under GABA-transaminase assay conditions is 0.0046 μmol/ml, which amounts to only 3.0% of the mean NADH production during 2 h of incubation. The use of the fluorimetric (measurement of NADH) and the radiochemical method (measurement of SSA + succinate) gave identical results, which also indicates that SSA accumulation is negligible. It is concluded that the excess of SSA-dehydrogenase over GABA-transaminase in homogenates is sufficient to prevent SSA accumulation during the GABA-transaminase assay. A large difference with both methods was only found in the kidney, indicating that SSA was accumulated in considerable amounts. Therefore, only the radiochemical method can be used in this tissue.

An important aspect of the coupled enzyme method described here is that it is assumed that the continuous removal of the intermediate SSA prevents the backwards reaction. This assumption is only valid if the affinity of SSA for the dehydrogenase is considerably higher than its affinity for GABA-transaminase. Evidence that the affinity of SSA for GABA-transaminase is relatively high will be presented in chapter 2.7. Studies of MAITRE et al. (1975) indicated that the

affinity of SSA for the backward reaction was comparetively low. However, the affinity of SSA for the transaminase was studied under conditions where inhibition by the co-substrate glutamate might strongly interfere. This backward reaction may become especially relevant if the affinity of SSA for the dehydrogenase is affected by some particular treatment, as has already been discussed in the case of the effect of NaCl on GABA-transaminase.

Of the GABA shunt enzymes SSA-dehydrogenase has received little attention. The reported high affinity of SSA for the dehydrogenase and the reports showing that an excess of SSA-dehydrogenase is present in the brain when compared with GABA-transaminase, are expecially relevant for the functioning of the GABA-shunt *in vivo* (BAXTER, 1972) as well as for the GABA-transaminase assay reported here. We have studied the kinetics of SSA-dehydrogenase using the integrated Henri-Michaelis-Menten method. The use of the double reciprocal method was not allowed since the decrease in substrate concentration during incubation was substantial, especially at low substrate concentration. As a concentration of 0.44 mg tissue/ml, a biphasic curve was found in the absence of salt (Fig. 2.3.10). This phenomenon is referred to in the literature as substrate inhibition (EMBREE & ALBERS, 1964; KAMMERAAT & VELDSTRA, 1968). Addition of NaCl at a concentration of 0.44 mg tissue/ml had two effects: antagonism of substrate inhibition and lowering of affinity of SSA for the dehydrogenase. Both phenomena can be explained by a decreased binding of SSA in the presence of NaCl mediated directly via competition with SSA or mediated indirectly via conformatial changes in the enzyme. Activation of SSA-dehydrogenase by addition of salt was also demonstrated by others (PITTS & QUICK, 1965). At a oncentration of 4.44 mg tissue/ml the affinity of SSA for SSA-dehydrogenase was comparatively low and the substrate inhibition observed at low tissue concentration could not be detected. However, it might be that this phenomenon only occurs at substrate concentrations above 0.3 mM. No conclusions could be drawn concerning the effect of NaCl at this tissue concentration since the highest SSA concentration used was equal to the apparent K_m. The results suggest that SSA-dehydrogenase might exist in two different states of aggregation: one with a high and one with a low affinity for the substrate. Some evidence in favour of this hypothesis can be found in recent reports in which it was shown that SSA-dehydrogenase obtained from *pseudomonas* is able to form dimers and to dissociate into monomers or even subunits (ROSEMBLATT et al., 1973; CALLEWAERT et al., 1973). It is possible that an increase in tissue and enzyme concentration might cause a shift in the association-dissociation equilibrium towards association, thus causing the observed decrease in affinity of SSA for the dehydrogenase. Some other evidence has been presented indicating the presence of two species of human brain SSA-dehydrogenase, since a biphasic double reciprocal curve with varying NAD concentration was found (EMBREE & ALBERS, 1964). Indirect support for the effect of tissue concentration on the

affinity of SSA for the dehydrogenase can be found in the study of KAMMERAAT (1966). A K_m of SSA for the dehydrogenase of 4 μM using 0.33 mg tissue/ml was observed. An increase of tissue concentration from 0.33 to 0.5 mg tissue/ml resulted in a detectable deviation from linearity for the time course. This was in conflict with the observed high affinity of SSA for the dehydrogenase, suggesting that the increase in tissue concentration caused a decrease of affinity of SSA for the dehydrogenase.

The presence of a high affinity form of SSA-dehydrogenase is of crucial importance for a valid GABA-transaminase assay, since it must prevent accumulation of SSA. Some evidence can be found demonstrating that a high as well as low affinity form of SSA-dehydrogenase are present during the GABA-transaminase assay: firstly, addition of NaCl or increase of tissue concentration resulted in a progressively decreasing rate of NADH formation by SSA-dehydrogenast under GABA-transaminase conditions (Fig. 2.3.12) and secondly, a comparison of the radiochemical and fluorimetric methods suggests that no significant accumulation of SSA occurred during the GABA-transaminase reaction. The extrapolation studies indicate that the steady-state concentration of SSA is about 5 μM, supporting the hypothesis that a high affinity form of SSA-dehydrogenase with a K_m in the order of 5 μM is active during GABA-transaminase assay at a concentration of 2.22 mg/kg. However, the affinity studies for SSA-dehydrogenase indicate that with a concentration of 2.22 mg tissue/ml the K_m is somewhere between 19.3 and 90.7 μM. Thus all control studies indicated that SSA accumulation did not occur during GABA-transaminase. SSA-dehydrogenase is about twice as active as GABA-transaminase. Accumulation of SSA to a level in the order of the K_m of SSA for the dehydrogenase is therefore sufficient to convert all SSA formed into succinate.

The results in Table 2.3.4 indicate that the K_m is almost one order of magnitude higher than the one suggested from the coupled GABA-transaminase assay experiments. These results can be explained by postulating the existance of two forms of SSA-dehydrogenase, one with a high and one with a low affinity for SSA. The prevention of accumulation of SSA by a high affinity form of SSA-dehydrogenase is in accordance with this theory and provides additional evidence for it.

Theoretical models describing the kinetics of a system in which the substrate is converted by two enzymes with different K_m's (or, as suggested here, by two forms of the same enzyme) show that only very small deviations from linearity occur when double reciprocal curves are used. Therefore, it is not possible to measure both kinetic parameters by some form of extrapolation, since the presence of the two kinetically distinct forms of the enzyme will result in an intermediate K_m value. The existance of substrate inhibition is another complicating factor in the study of the kinetics of SSA-dehydrogenase.

In conclusion, the use of the coupled enzyme method for the assay of GABA-

transaminase activity seems justified under the conditions described since GABA-transaminase is rate limiting and since the high affinity form of SSA-dehydrogenase prevents SSA accumulation. Therefore, if used under proper incubation conditions, it offers the possibility to measure GABA-transaminase activity in small brain samples without further extensive purification of the coupling enzyme.

2.4. THE EFFECT OF NaCl ON SSA-DEHYDROGENASE

The effect of NaCl on SSA-dehydrogenase was investigated to explore whether the effect of NaCl was similar to that on GABA-transaminase and whether the specific effect of NaCl observed on SSA-dehydrogenase (see chapter 2.3) may support the hypothesis that the dehydrogenase has a regulatory function in the degradation of GABA.

2.4.1. Comparison of the effect of NaCl on GABA-transaminase and SSA-dehydrogenase

In these experiments SSA-dehydrogenase was studied under GABA-transaminase assay conditions using SSA as a substrate to allow a comparison between the effects of NaCl on SSA-dehydrogenase and GABA-transaminase. The curve for GABA-transaminase obtained from the values of Fig. 2.3.4 was included for this purpose. GABA-transaminase was assayed using the coupled enzyme method.

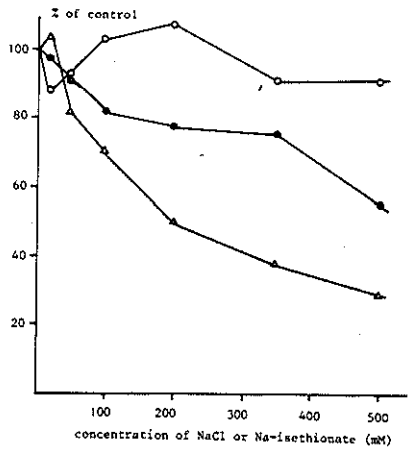


Fig. 2.4.1. The effect of NaCl on GABA-transaminase and SSA-dehydrogenase. The incubation mixture contained: 50 mM TRIS-HCl, pH 8.5; 2.0 mM 2-oxoglutaric acid; 20 mM 2-mercaptoethanol; 1.1 mM NAD; 6.67 mg tissue/ml and 3.0 mM GABA for the GABA-transaminase assay and 0.3 mM SSA for the SSA-dehydrogenase assay. Incubation time was 10 (–○–○–) or 30 (–●–●–) min for SSA-dehydrogenase and 120 min for GABA-transaminase (–△–△–). SSA-dehydrogenase was assayed with varying amounts of NaCl, while for GABA-transaminase the mean values for NaCl or Na-isethionate are plotted. Control values for GABA-transaminase or SSA-dehydrogenase activities were 33.6 μMol/g/h (n = 2) or 60.4 ± 6.2 μMol/g/h (n = 3), respectively.

The results in Fig. 2.4.1 indicate that with 6.7 mg tissue/ml no inhibition of SSA-dehydrogenase was observed after an incubation time of 10 min. By increasing the incubation time a progressively increasing inhibition by NaCl was observed. With 200 mM NaCl a 20% inhibition was observed, which is similar to that observed in chapter 2.3, Fig. 2.3.13. For GABA-transaminase a progressively increasing inhibition was observed resulting in 50% inhibition at 200 mM NaCl or Na-isethionate. As a consequence, the ratio of GABA-transaminase and SSA-dehydrogenase activity decreased with NaCl addition from 0.56 in the absence of NaCl to 0.16 in the presence of NaCl.

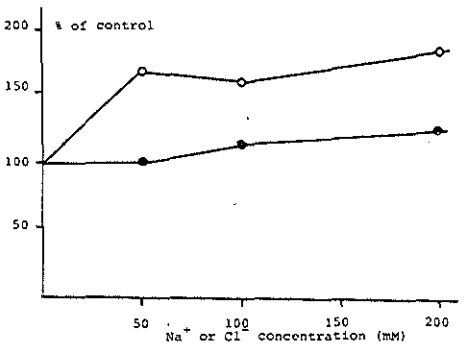


Fig. 2.4.2. Effect of Na^+ and Cl^- on SSA-dehydrogenase activity. The incubation mixture contained: 0.04-0.08 mM SSA formed by pre-incubation with GABA and 2-oxoglutaric acid in the absence of NAD; 50 mM TRIS-HCl, pH 8.5; 3.0 mM GABA; 2.0 mM 2-oxoglutaric acid; 20 mM 2-mercaptoethanol; 0.16 mM NAD and 2.22 mg tissue/ml. Mixtures of NaCl, Na-isethionate and choline chloride were prepared such that while keeping the ionic strength at 0.22 the concentration of Na^+ or Cl^- was 50, 100 or 200 mM. The following mixtures were used: No NaCl; 200 mM choline chloride or Na-isethionate; 50 mM NaCl with 150 mM choline chloride or Na-isethionate; 100 mM NaCl with 100 mM choline chloride or Na-isethionate. Control activity was 38.9 μMol/g/h, while with 200 mM choline chloride or Na-isethionate activities were 18.4 and 27.1 μMol/g/h, respectively. Experimental points: varying Na^+: (–○–○–); varying Cl^- (–●–●–), for two experiments.

2.4.2. The effect of Na^+ and Cl^- on SSA-dehydrogenase activity

For these particular experiments GABA and 2-oxoglutaric acid were pre-incubated for 1 h with homogenate (6.67 mg tissue/ml) without NAD. Thereafter, the incubation was continued with two vol of a mixture containing NAD and different amounts of NaCl, choline chloride and Na-isethionate as indicated in the legend of Fig. 2.4.2. SSA-concentration, formed using this procedure, is

about 0.04 to 0.08 mM as calculated from SSA-formation in the absence of NAD described in chapter 2.7. Because GABA-transaminase is still active the decrease of SSA-concentration during incubation is negligible. As a result linear time courses were obtained indicating that the decrease of substrate concentration during incubation did not influence the rate of NADH formation. The activity for SSA-dehydrogenase was calculated from the linear time courses using 0 to 30 min of incubation. Both ionic strength and Na^+ had pronounced effects on SSA-dehydrogenase activity. By keeping ionic strength constant by adding additional amounts of choline chloride or Na-isethionate to varying amounts of NaCl as indicated in the legend of Fig. 2.4.2., a 75% activation by Na^+ was unmasked. This activation by Na^+ is optimal at the physiologically relevant concentration of 50 mM NaCl or lower. The effect of Cl^- (20% activation) is only minimal. The effect of ionic strength was demonstrated by a comparison of the effect of 100 mM NaCl (ionic strength: 0.12) and the effect of a mixture of 100 mM choline chloride and 100 mM Na-isethionate (100 mM NaCl, ionic strength: 0.22). The mixture with 100 mM NaCl and ionic strength 0.22 was 35% inhibited when compared with 100 mM NaCl with ionic strength 0.12. Similar effects of Na^+ were found as described above by comparing the activity of SSA-dehydrogenase in the presence of similar concentrations of NaCl, choline chloride or Na-isethionate (not shown). A 40% activation by Na^+ was observed under these conditions using 50 to 100 mM NaCl or 50 to 100 mM Na-isethionate, respectively.

2.4.3. The effect of NaCl in the presence of glycylclycine-KOH and TRIS-HCl

From experiments described in chapter 2.3 (Table 2.3.1) with glycylglycine-KOH and TRIS-HCl buffers it was concluded that the low activity for GABA-transaminase in glycyglycine-KOH was caused by inhibition of SSA-dehydrogenase in the presence of this buffer. Addition of TRIS or salt (NaCl or KC) increased GABA-transaminase considerably. Therefore, the effect of 200 mM NaCl on SSA-dehydrogenase was further investigated in TRIS-HCl, TRIS-glycyl- and the results are summarized in Table 2.4.1. The results in Fig. 2.4.3 demonstrate the strong inhibitory effect of glycylglycine on SSA-dehydrogenase which can be observed both in the absence (a) and presence (b) of 200 mM NaCl. SSA-dehydrogenase is activated considerably in the presence of glycylglycine-KOH containing buffers, while in TRIS-HCl buffer alone a 25% inhibition was observed. The effect of NaCl on SSA-dehydrogenase in the presence of glycylglycine-KOH was not significant because of the large variability in SSA-dehydrogenase activity observed in the presence of this buffer. Experiments under similar conditions using the spectrophotometrical method to measure NADH formation (see chapter 2.1.5) and rat brain SSA-dehydrogenase indicated that

sometimes up to 400 mM NaCl was needed to activate the enzyme in the presence of glycylglycine-KOH buffer. In addition, the presence of a considerable lag-time might contribute to the observed large variability (Fig. 2.4.3).

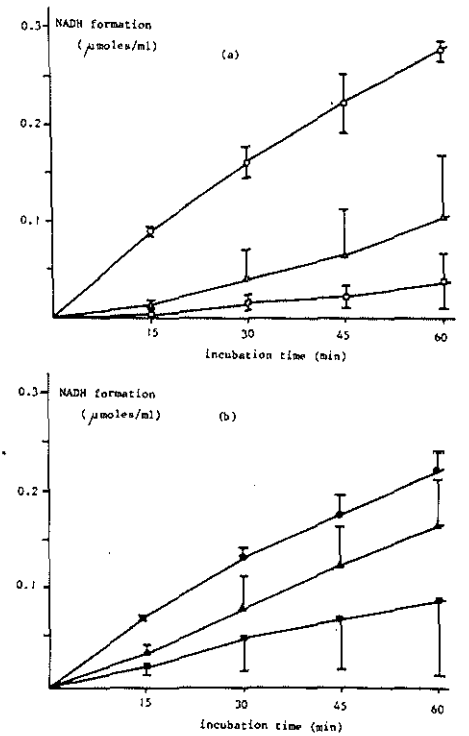


Fig. 2.4.3. The effect of NaCl in the presence of glycylglycine-KOH and TRIS-HCl buffers. The incubation mixture contained: 0.3 mM SSA; 1.1 mM NAD; 2.0 mM 2-oxoglutaric acid; 20 mM 2-mercapto-ethanol; 6.67 mg tissue/ml, dissolved in the following buffers: 50 mM TRIS-HCl, pH 8.5: (○, ●); 50 mM TRIS-HCl + 30 mM glycylglycine-KOH buffer, pH 8.5: (△, ▲) and 30 mM glycylglycine-KOH, pH 8.5: (□, ■). The experiments under (a) were performed without NaCl, while the experiments under (b) were performed in the presence of 200 mM NaCl. Results are the mean ± S.E. of three experiments. Experimental points: ○, △, □: control; ●, ▲, ■: in the presence of 200 mM NaCl.

2.4.3. Discussion

As suggested in chapter 2.3, NaCl might have an activating effect on SSA-dehydrogenase activity. There are, however, many disturbing factors such as the effect of ionic strength on the affinity of SSA for the dehydrogenase and the

observed antagonism of substrate inhibition. The experiments described in Fig. 2.4.1 demonstrate that 200 mM NaCl has differential effects on GABA-transaminase and SSA-dehydrogenase activity at the same tissue concentration. No effect of NaCl on SSA-dehydrogenase activity was observed with a short incubation time, while increasing the incubation time resulted in inhibition of NADH formation by NaCl. This inhibition is probably caused by the decrease of affinity with increasing NaCl (see table 2.3.4, chapter 2.3), which results in a decrease of the affinity of SSA for the dehydrogenase. As a consequence the reaction will not be zero order with respect to substrate resulting in a deviation of linearity of the time course as observed and discussed in chapter 2.3. This might explain the observed variation of the effect of NaCl with incubation time (Fig. 2.4.1). This variable was eliminated by using the incubation conditions described in the legend of Fig. 2.4.2, where tissue concentration was reduced to 2.22 mg tissue/ml, while GABA-transaminase was active by synthesizing SSA preventing the decrease of SSA-concentration during the course of the reaction. This resulted in linear time courses suggesting that substrate concentration was not a variable in these experiments during the course of the reaction. The second variable, viz. ionic strength, was controlled by addition of different amounts of choline chloride to keep te concentration of Cl^- as well as the ionic strength at a constant level. Under these conditions a 75% activation of SSA-dehydrogenase activity by Na^+ was revealed. These results are in agreement with the observed effects of NaCl on the kinetic parameters of SSA-dehydrogenase where V_m was increased at 4.44 mg tissue/ml (chapter 2.3, Table 2.3.4). This might also indicate that the two effects of NaCl on SSA-dehydrogenase, viz. increase of V_m and decrease of K_m, are mediated by Na^+ or ionic strength, respectively.

The observed increase of SSA-dehydrogenase activity in the presence of glycylglycine-KOH by NaCl addition might be related to the effect of NaCl on substrate inhibition (chapter 2.3). Since glycylglycine has structural similarities with SSA and inhibition of SSA-dehydrogenase by SSA as well as glycylglycine was antagonized by NaCl addition, the same binding sites on SSA-dehydrogenase might be involved in this effect of NaCl.

These results as well as the results described in chapter 2.3 demonstrate that the effect of NaCl on rat brain SSA-dehydrogenase is complex. Therefore, these phenomena were studied in an experimental model for the rat brain SSA-dehydrogenase, viz. the bacterial SSA-dehydrogenase present in the partially purified and commercially available "GABASE" which is prepared from the bacterium *pseudomonas fluorescence.*

2.5. THE EFFECT OF DPA, MONOVALENT AND BIVALENT CATIONS ON *PSEUDOMONAS* SSA-DEHYDROGENASE

The experiments with rat brain SSA-dehydrogenase indicated that the effect of NaCl on SSA-dehydrogenase was complex and involved a coincidence of several independent effects. Since the *pseudomonas* enzyme was different from the rat brain enzyme in a number of aspects and therefore might have a less complex nature, the properties of this enzyme were studied. If some of the properties of the bacterial enzyme were comparable to those of the rat brain enzyme, then the *pseudomonas* enzyme might be useful as a model for brain SSA-dehydrogenase.

2.5.1. Optimal incubation conditions

Pseudomonas SSA-dehydrogenase was obtained from Worthington as "GABASE" and was used without further purification. Initial experiments indicated that the presence of 2-mercaptoethanol was important for stabilization of the enzyme in sucrose-Triton-TRIS as well as for optimal activity of the enzyme. V_m but not K_m was affected, while optimal activities were obtained with 10 mM 2-mercaptoethanol. From experiments with varying amounts of NADP it appeared that SSA-dehydrogenase was saturated with 0.5 mM NADP.

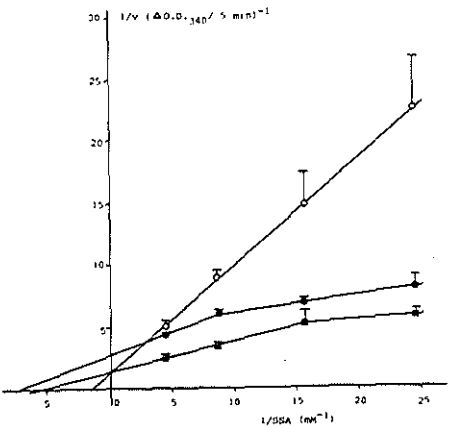


Fig. 2.5.1. The effect of the sodium salt of DPA on SSA-dehydrogenase. The incubation mixture contained: 50 mM TRIS-HCl, pH 8.5; 0.5 mM NADP; 10 mM 2-mercaptoethanol; 0.44 mg GABASE/ml and varying amounts of SSA. Experimental points: control (–●–●–); 10 mM Na-isethionate (–■–■–); 10 mM Na-DPA (–○–○–). The experiments were performed at 38°C for 5 min.

2.5.2. Effect of DPA

The effect of DPA was studied using different amounts of SSA. The results in Fig. 2.5.1 show that both K_m and V_m were effected by DPA. Because together with DPA also 10 mM Na^+ was introduced, the effect of Na-isethionate was studied. It appeared that in the presence of Na^+ the V_m but not the K_m was considerably increased. Comparison of the effect of DPA using the curve for 10 mM Na-isethionate as a control indicated that DPA was a competitive antagonist that decreased the K_m in the presence of Na^+. V_m was not affected, suggesting that the effect of DPA on V_m when compared with a control without Na^+ was caused by the Na^+ present in the DPA.

2.5.3. Kinetics of *Pseudomonas* SSA-dehydrogenase and the effect of Na^+

The experiments with DPA indicated that SSA-dehydrogenase was not fully active under control conditions, but could be activated considerably by addition of Na^+. Also the double reciprocal curve seemed to be biphasic. Therefore, the effect of substrate concentration was studied in the presence, as well as in the absence of 10 mM Na-isethionate. The results in Fig. 2.5.2 indicate that the

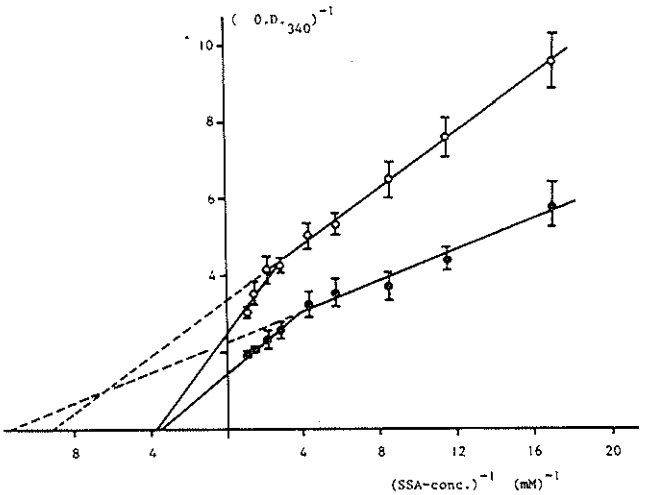


Fig. 2.5.2. The effect of Na-isethionate on kinetic parameters of SSA-dehydrogenase. The incubation mixture contained: 50 mM TRIS-HCl, pH 8.5; 0.04 to 1 mM SSA; 10 mM 2-mercaptoethanol; 0.5 mM NADP and 0.44 mg GABASE/ml. Experimental points: control (–○–○–), n = 4; 10 mM Na-isethionate (–●–●–), n = 3. Results are presented as the mean ± S.E.

double reciprocal curve for SSA-dehydrogenase is biphasic in the presence, as well as in the absence of Na-isethionate. This suggests that two forms of SSA-dehydrogenase or two different enzymes catalyzing the same reaction are present in the GABASE preparation. Furthermore, the results demonstrate that 10 mM Na-isethionate increased the V_m for both forms while the K_m remained relatively unaffected (Table 2.5.1). Using the method of SPEARS et al. (1971) to approximate the kinetic constants of these enzymes or enzymatic forms, better estimates were calculated for one experiment. This resulted in K_m values of 40 and 900 μM and V_m values of 0.180 and 0.388 for the high or low affinity form, respectively, in the absence of Na-isethionate. This indicates that about 70% of SSA-dehydrogenase was present in the low affinity form. Both forms were apparently activated in the presence of Na^+.

2.5.4. The effect of varying Na^+ on SSA-dehydrogenase activity

The effect of Na^+ on SSA-dehydrogenase is shown in Fig. 2.5.3. Maximal activation of SSA-dehydrogenase was obtained between 25 and 100 mM NaCl, while with increasing NaCl further activity decreased to control levels at 500 mM NaCl.

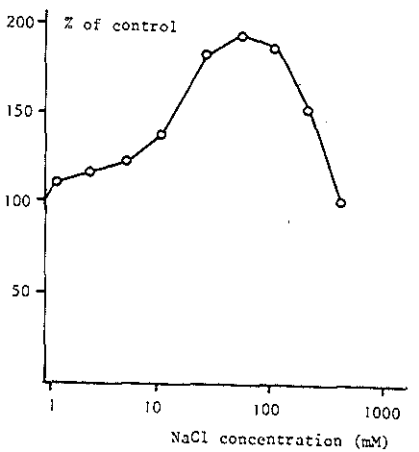


Fig. 2.5.3. The effect of NaCl on SSA-dehydrogenase activity. The incubation mixture contained: 50 mM TRIS-HCl, pH 8.5; 10 mM 2-mercaptoethanol; 0.5 mM NADP; 0.23 mM SSA; 0.44 mg GABASE/ml and varying amounts of NaCl. The incubations were performed at 38°C for 5 min.

Table 2.4.1.

Extrapolated kinetic parameters for SSA-dehydrogenase

	K_m (μM)		V_m (Δ O.D.$_{340}$/5 min)	
	control	10 mM Na^+	control	10 mM Na^+
High affinity form	116 ± 7	88 ± 2	0.282 ± 0.021	0.445 ± 0.043
Low affinity form	308 ± 19	292 ± 59	0.381 ± 0.029	0.695 ± 0.046

The experimental details are described in the legend of Fig. 2.4.2. Extrapolations for the low affinity form are based on values of SSA concentrations between 0.93 and 0.23 mM at 10 mM Na-isethionate and 0.93 and 0.35 mM in the absence of Na-isethionate. N.B.: The V_m value obtained from extrapolation at high substrate concentration is the sum of V_m values for both forms of SSA-dehydrogenase.

2.5.5. The effect of different monovalent cations on SSA-dehydrogenase activity

SSA-dehydrogenase was activated by monovalent cations in accordance with their ionic radius (HOLLEMAN, 1959) as shown in Fig. 2.5.4. Optimal activation was obtained with 25 mM K^+, by which the enzyme was activated to about 275% of control values. Also NH_4^+, having an ionic radius of 1.27 Å, fitted nicely in the curve with an activity of 232.9 ± 4.5% (n = 3) as compared to controls.

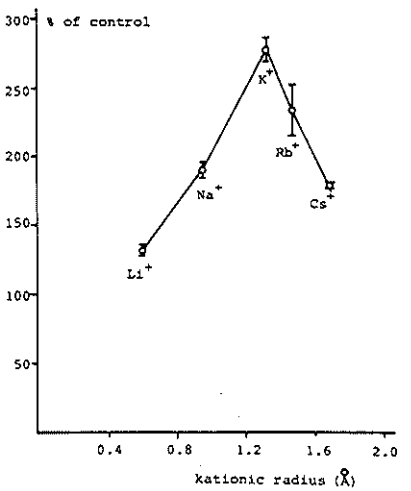


Fig. 2.5.4. The effect of ionic radius on SSA-dehydrogenase activation by bivalent cations. Experimental details are as described in the legend of Fig. 2.5.3. The concentration of the chloride salts was 25 mM. The results are the mean ± S.E. for 3 or 4 experiments.

Table 2.5.2.

Effect of different anions and ionic strength on SSA-dehydrogenase activity

addition	% of control activity	(n)
buffer	100.0 ± 3.1	(4)
choline chloride	99.5 ± 1.2	(4)
sucrose	101.0 ± 1.3	(4)
NaCl	183.1 ± 8.5 *	(4)
Na-isethionate	188.1 ± 6.1 *	(4)
KCl	277.7 ± 8.6 *	(4)
KJ	181.5 ± 3.4 **	(3)

For experimental details see the legend of Fig. 2.5.3. All additions were to a final concentration of 25 mM, except for sucrose which was 50 mM to obtain an iso-osmotic control for the salt additions to demonstrate the effect of ionic strength. * = $p < 0.001$ when compared with buffer, sucrose or choline chloride. ** = $p < 0.001$ when compared with KCl

The effects of anions and ionic strength are demonstrated in Table 2.5.2. The addition of either 25 mM choline or sucrose was not inhibitory since the activity in the presence of choline chloride or 50 mM sucrose was similar to control values. In addition no difference exists between the activatory effect of NaCl when compared with Na-isethionate indicating that Cl^- did not contribute to the observed activation by the salts presented in Fig. 2.5.4. However, replacement of Cl^- by J^- resulted in a significant reduction of the activation by KCl when compared with KJ. This indicates that J^- has an effect of its own or may modify the activation of SSA-dehydrogenase by K^+.

2.5.6. Effect of varying Ca^{2+} on SSA-dehydrogenase activity

The effect of Ca^{2+} concentration is shown in Fig. 2.5.5. Maximal activation was obtained at 5 mM, though at this concentration of Ca^{2+} the time course of NADPH formation was linear for a shorter time period than for lower Ca^{2+} concentrations. From these results an ED_{50} was calculated of 0.5 mM Ca^{2+}.

2.5.7. The effect of different bivalent cations on SSA-dehydrogenase activity

The effect of bivalent cations was studied using a concentration of 2.5 mM. Maximal activation was obtained in the presence of Mg^{2+} or Ca^{2+}, while a further increase in ionic radius decreased the observed activation by bivalent cations (Fig. 2.5.6).

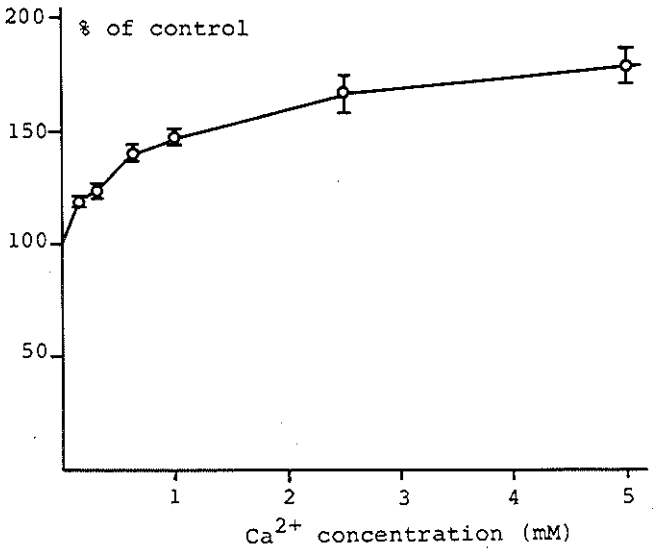


Fig. 2.5.5. The effect of Ca^{2+} on SSA-dehydrogenase activity. The incubation conditions are as described in Fig. 2.5.3., instead that varying amounts of $CaCl_2$ were used here. The results are the mean ± S.E. for 3 experiments.

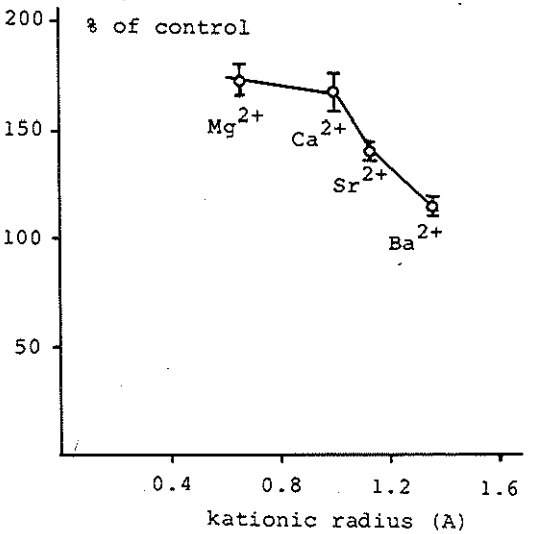


Fig. 2.5.6. The effect of ionic radius on SSA-dehydrogenase activation by bivalent cations. For experimental details see the legend of Fig. 2.5.3. The concentration of the salts is 2.5 mM. The results are the mean ± S.E. for 3 experiments. All activations are significant when compared with control ($p < 0.001$).

Table 2.5.4.

Interactions between the effects of KCl, $CaCl_2$ and substrate administration

Pretreatment	(N)	Initial activity	activity after 10% SSA conv.	$CaCl_2$ addition	KCl addition
None	(4)	100.0 ± 4.8	100.0 ± 4.8	85.0 ± 3.3**	163.9 ± 18.6***
25 mM KCl	(4)	264.5 ± 4.3*	202.2 ± 12.9*	132.7 ± 21.6**	–
2.5 mM $CaCl_2$	(4)	155.6 ± 6.2*	132.2 ± 2.5*	–	138.3 ± 11.4***
25 mM KCl + 2.5 mM $CaCl_2$	(3)	233.4 ± 3.2*	163.3 ± 19.1*	–	

* $p < 0.001$ as compared to activity in non-treated controls.
** $p < 0.05$ as compared to control after 10% SSA conversion (Ca^{2+} inhibition).
*** $p < 0.05$ as compared to control after 10% SSA conversion (K^+ activation).

The incubation mixture contained: 50 mM Tris-HCl, pH 8.5; 0.5 mM NADP; 10 mM 2-mercaptoethanol; 0.44 mg tissue/ml and 0.232 mM SSA. Additions of KCl or $CaCl_2$ before or after administration of SSA were performed with 10 µl additions in a total volume of 1,125 ml. Pretreatments refer to experiments where the bivalent and monovalent cations were already present during preincubation in the absence of SSA. Additions refer to experiments where the cations were administered after starting the reaction with SSA at a time where an amount of NADPH equivalent to an optical density of 0.150 (about 10% conversion of SSA) had been formed. The rates of NADPH formation after treatment with these cations were compared with control rates under similar conditions. Control activity was 0.392 ± 0.019 (4) $\Delta O.D._{340}/5$ min, which is equal to an activity of 1660 µMol/g dry wt/h at 38°C.

2.5.8. Interactions between the activation by KCl and $CaCl_2$ and substrate addition

Since monovalent and bivalent cations activated SSA-dehydrogenase an investigation was made to see whether the same site on the enzyme or the same mechanism was involved in the observed activation by both types of cations. Therefore, the effect of combined addition of KCl and $CaCl_2$, as described in the legend of Table 2.5.3, was compared with the effect of either salt alone. The activities in the first column refer to experiments where KCl, $CaCl_2$, or KCl and $CaCl_2$ together were added to the incubation mixture. Both $CaCl_2$ and KCl activated SSA-dehydrogenase, while the combined treatment resulted in an intermediate activation. The experiments in the second column refer to the same incubations, while the activity of SSA-dehydrogenase was measured after 10% conversion of SSA. The activations are somewhat less but the pattern is similar to that observed in the first column measuring the initial rate. The third and fourth column refer to experiments where $CaCl_2$ or KCl were added to incubation mixtures already containing no salt or 25 mM KCl or 2.5 mM $CaCl_2$. At a time when 10% conversion of SSA was observed KCl or $CaCl_2$ were added and the initial rate after addition of the salts was noted. Therefore, the results of the third and fourth column have to be compared to the activations measured in the second column. The results in the third column indicate that addition of $CaCl_2$ after starting the reaction had an inhibitory effect on SSA-dehydrogenase instead of an activation observed when $CaCl_2$ was added before administration of SSA as shown in the first column. The activity of SSA-dehydrogenase was 132.2% when $CaCl_2$ was added before SSA, while an activity of 85.0% was observed when $CaCl_2$ was added after SSA administration. Also KCl addition was slightly diminished when the activity with KCl administration before SSA-administration (202.2%) was compared to the activity observed with KCl administration after SSA-addition (163.9%). The addition of $CaCl_2$ after SSA administration to a KCl containing incubation mixture was also inhibitory (activity is 132.7%) when compared with a KCl containing medium (activity is 202.2%). Therefore, administration of $CaCl_2$ after SSA invariably results in an inhibition of SSA-dehydrogenase activity, while an activation is observed with $CaCl_2$ addition before SSA administration in the absence of KCl. Whereas an activation of SSA-dehydrogenase activity was observed by KCl administration to control incubation mixtures, no effect was observed after administration of KCl to $CaCl_2$ containing incubation mixtures (no KCl: 132.2% and with KCl: 138.3%). Therefore, the presence of $CaCl_2$ abolished the activation observed after administration of K^+.

2.5.9. Discussion

The experiments with DPA confirm the results of HARVEY et al. (1975) and ANLEZARK et al. (1976) that DPA is a competitive inhibitor of SSA-dehydrogenase. Using the method suggested by SEGEL (1975) a K_i for DPA of 3.9 mM was calculated, based upon measurements with the low affinity form of SSA-dehydrogenase having a K_m of about 0.9 mM. The observed effect of DPA on the V_m could be ascribed to an activatory effect of the sodium ions present in the administered DPA giving a final Na^+-concentration of 10 mM in the incubation mixture. This was demonstrated by the increase of V_m observed after incubation in the presence of 10 mM Na-isethionate with varying amounts of SSA, which was similar to that observed with the Na^+ containing DPA.
A biphasic double reciprocal curve was obtained using varying amounts of SSA, both in the presence, as well as in the absence of 10 mM Na-isethionate. This indicates that two forms of the same enzyme or two different enzymes are present in the bacterial GABASE preparation catalyzing the same reaction, but having quite different kinetic characteristics. Addition of 10 mM Na-isethionate resulted in nearly parallel effects on both parts of the biphasic double reciprocal curve. This suggests that two forms of the same enzyme, both activated by Na^+, were present instead of two different enzymes. The extrapolated values for K_m and V_m summarized in Table 2.5.1. are very rough approximates for the kinetic parameters of both forms of SSA-dehydrogenase. The method of SPEAR et al. (1971) was used to approximate these kinetic parameters, a method originally devised for deriving kinetic constants for two enzymes acting on the same substrate. With this curve fitting method K_m values of 40 μM and 0.9 mM were calculated. However, it must be kept in mind that curved reciprocal plots do not necessarily indicate the presence of multiple enzymes or multiple forms of the same enzyme. Multiple site enzymes having substrate binding sites of different affinities or multiple site enzymes that display negative cooperativity also yield curved reciprocal plots (SEGEL, 1975).
Using varying amounts of NaCl a maximal activation by 80% was obtained using 25-100 mM NaCl. A close relationship was observed between the extent of activation and the ionic radius of several monovalent cations. The results in Table 2.5.2. demonstrate that the anion (Cl^-) did not contribute to the observed activation of SSA-dehydrogenase. The results with varying SSA concentration in the presence and absence of Na-isethionate suggest that Na^+ and probably also the other monovalent cations increased V_m while having little effect on the K_m. The bivalent cation Ca^{2+} activated SSA-dehydrogenase by 60% at an optimal concentration of 2.5 mM and an ED_{50} value of 0.5 mM. A similar activation was obtained with Mg^{2+}, while a further increase in the ionic radius decreased the activatory effect of bivalent cations considerably. The experiments with $CaCl_2$ administration before or after SSA addition indicated that the activatory effect

of $CaCl_2$, observed when SSA was added after $CaCl_2$ addition, was reversed in an inhibition of SSA-dehydrogenase when the order of administration was opposite. A similar effect with KCl administration was not observed. This suggests that $CaCl_2$ and KCl, representing the bivalent or monovalent cations, respectively, have a different mode of action on SSA-dehydrogenase. The results with Na-isethionate indicate that the monovalent cation increased V_m without affecting K_m, while the effects of Ca^{2+} are closely associated with substrate binding. This suggests that Ca^{2+} may interfere with the affinity of SSA for the dehydrogenase or may cause a change of the low affinity form into the high affinity form. However, such a possible mode of action of Ca^{2+} remains to be investigated.

The experiments with rat brain SSA-dehydrogenase and GABA-transaminase indicated that salt addition had differential effects on these GABA degrading enzymes. Whereas addition of NaCl, KCl, choline chloride and Na-isethionate had a similar inhibitory effect of about 50% on rat brain GABA-transaminase (see chapter 2.3.1, Table 2.3.1), addition of NaCl activated SSA-dehydrogenase as demonstrated in chapters 2.3.2 and 2.4. In both studies NaCl increased SSA-dehydrogenase activity with about 70 to 80%, which is in close agreement with the results obtained with the bacterial SSA-dehydrogenase as observed in this chapter. The observation that two forms of SSA-dehydrogenase might be present in the bacterial preparation, both being activated by Na^+, is also in accordance with the results obtained in chapter 2.3, where a similar conclusion was reached for rat brain SSA-dehydrogenase. However, for rat brain SSA-dehydrogenase this conclusion was based on the effect of tissue concentration on the kinetic parameters of SSA-dehydrogenase. It is tempting to extend the observations with the bivalent cations to the rat enzyme. However, this must await experimental verification before bivalent cations can be implicated in the regulation of the degradation of GABA via SSA-dehydrogenase.

In conclusion, the experiments with rat brain and bacterial SSA-dehydrogenase demonstrate that both enzymes have a number of properties in common. However, they do not behave completely similarly, as demonstrated by the differential results with both enzymes for the double reciprocal curves with varying substrate concentrations. Therefore, the bacterial enzyme may offer the opportunity to explore some properties of bacterial SSA-dehydrogenase, which can be verified afterwards on the rat brain enzyme. This might be especially relevant for the study of a relationship between structure and activity of inhibitors of SSA-dehydrogenase and for the study of the effects of ions on the properties of SSA-dehydrogenase as demonstrated in this chapter. Thus, bacterial SSA-dehydrogenase may be valuable as a model for at least some aspects of the rat brain enzyme.

2.6. DIFFERENTIAL EFFECTS OF GABA-ANALOGUES ON GLUTAMATE DECARBOXYLASE, GABA-TRANSAMINASE AND SSA-DEHYDROGENASE ACTIVITY

The purpose of this study was twofold. Firstly, to study whether the GABA-shunt enzymes might be involved in the pharmacological action of 1-hydroxy-3-amino-pyrrolidone-2 (HA-966), 4-N-hydroxy-2,4-diaminobutyrate (NH-DABA) and Zn^{2+}. These compounds have been implicated in the development of experimental dyskinesias (BONTA et al., 1964; BONTA et al., 1971; IZUMI et al., 1973). Secondly, to establish whether it was possible to affect the GABA-shunt enzymes selectively.

2.6.1. Inhibition studies

Preliminary experiments indicated that some of the drugs used in this study interfered with PLP. Therefore glutamate decarboxylase and GABA-transaminase were assayed in the presence as well as in the absence of added PLP. The

Table 2.6.1.

The effect of drugs on GAD activity in the presence and absence of added PLP

Addition	concentration (mM)	activity (+PLP) (%)	activity (-PLP) (%)	$ratio_{PLP}$
None	–	100.0 ± 15.2	100.0 ± 13.2	0.307 ± 0.012
HA	5.0	99.0 ± 11.1	66.2 ± 3.2*	0.202 ± 0.006***
	0.5	99.0 ± 12.1	92.7 ± 13.6	0.284 ± 0.038
NH-DABA	5.0	25.9 ± 4.4**	4.4 ± 2.2***	0.065 ± 0.042***
	0.5	78.1 ± 10.0	15.7 ± 0.2***	0.064 ± 0.029**
Zinc sulphate	0.2	46.8 ± 2.7*	44.0 ± 11.0*	0.285 ± 0.049
	0.02	98.0 ± 13.1	76.8 ± 2.2	0.259 ± 0.031
p-HBA	0.1	92.9 ± 14.8	94.5 ± 2.2	0.327 ± 0.052
	0.01	97.9 ± 10.8	95.6 ± 8.8	0.299 ± 0.011
DABA	5.0	88.6 ± 6.1	68.1 ± 5.6*	0.234 ± 0.012*

The activity was measured in the presence or in the absence of 0.4 mM PLP. The incubation mixture contained 9.0 mM L-glutamate; 50 mM K-PO_4-buffer, pH 6.5; 20 mM 2-mercaptoethanol; 0.5 μCi DL-1-^{14}C-glutamic acid and 12.5 mg tissue (wet weight)/ml. The reaction was performed at 38°C for one hr. The $ratio_{PLP}$ represents the ratio of GAD activity without and with 0.4 mM added PLP. Control values for GAD activity were: 29.7 ± 4.5 μmoles/g tissue/hr in the presence and 9.1 ± 1.2 μmoles/g tissue/hr in the absence of added PLP. The results are the Mean ± SE for three experiments.
Significance of the inhibitions was studied using Student's t-test. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$.

possible interference of the drugs with PLP was studied by measuring the ratio of enzyme activity with and without PLP. A control ratio of 0.307 ± 0.012 was found for glutamate decarboxylase using 0.4 mM PLP, while a ratio of 1.034 ± 0.090 was found for GABA-transaminase. The latter experiments were performed using 0.1 mM PLP, since a higher concentration of PLP might inhibit the transaminase (BAXTER & ROBERTS, 1961). Apparently, GABA-transaminase is not affected by PLP addition, while glutamate decarboxylase was activated about threefold.

The results in Table 2.6.1. demonstrate that glutamate decarboxylase was inhibited by 5 mM HA-966 in the absence of added cofactor. Addition of PLP prevented the inhibitory effect of HA-966. Its aliphatic congener NH-DABA Inhibited glutamate decarboxylase activity almost completely in the absence of added cofactor, while PLP addition only partially restored glutamate decarboxylase activity. Zinc ions were equally effective in the presence or absence of added PLP. 2,4-diaminobutyrate (DABA) was only slightly inhibitory in the absence of added PLP, while decarboxylase activity was not affected by p-hy-

Table 2.6.2.

The effect of drugs on GABA-transaminase and SSA-dehydrogenase activity

Addition	concentration (mM)	GABA-T activity (%)	SSA-DH activity (%)
None	–	100.0 ± 7.6	100.0 ± 4.9
HA-966	5.0	92.1 ± 2.2	92.4 ± 5.1
	0.5	97.1 ± 4.5	95.6 ± 1.5
NH-DABA	5.0	6.1 ± 0.2***	28.3 ± 0.4***
	0.5	47.6 ± 8.8**	95.5 ± 3.0
Zinc sulphate	0.2	46.9 ± 13.7**	35.8 ± 4.4***
	0.02	80.0 ± 1.2*	84.8 ± 4.8*
p-HBA	0.1	5.9 ± 1.4***	17.5 ± 0.6***
	0.01	47.0 ± 7.8**	70.5 ± 7.4*
DABA	5.0	66.0 ± 1.9**	91.1 ± 6.9

GABA-T activity was measured with 2.22 mg tissue/ml and with an incubation time of 2 hrs. The incubation mixture contained: 50 mM Tris-HCl, pH 8.5; 3.0 mM GABA; 2.0 mM 2-oxoglutaric acid, 20 mM 2-mercaptoethanol, 0.9 mM NAD and drugs at the concentration indicated. Results represent the Mean ± SE for three experiments. Control GABA-T activity, measured at 22°C, was 29.3 ± 2.2 μmoles/g tissue/hr. SSA-DH activity was measured with 0.44 mg tissue/ml with an incubation time of 2 hrs. The incubation mixture contained: 50 mM Tris-HCl, pH 8.5; 0.25 mM NAD; 10 mM 2-mercaptoethanol; 0.3 mM SSA and effectors at the concentration indicated. Results are the Mean ± SE for three experiments. Control value for SSA-DH activity was 52.8 ± 2.6 μmoles/g tissue/hr.

Significance was tested using Student's t-test.

* = $p < 0.05$; ** = $p < 0.0125$; *** = $p < 0.001$.

droxybenzaldehyde (p-HBA). The ratio of glutamate decarboxylase activity with or without added PLP was significantly reduced by HA-966, NH-DABA and DABA.

The results in Table 2.6.2 show that GABA-transaminase activity was not changed by HA-966, while it was strongly inhibited by NH-DABA. Zinc ions were moderately inhibitory at 0.2 mM, while a small effect was observed at 0.02 mM. p-HBA inhibited GABA-transaminase activity at both concentrations studied, while DABA was slightly inhibitory at 5 mM. No significant effect was observed on the ratio of GABA-transaminase activity with or without added PLP.

The inhibition studies for SSA-dehydrogenase were performed with a tissue concentration of 0.44 mg tissue/ml. Similar results were obtained with 2.22 mg tissue/ml indicating that the kinetic properties of the enzyme did not influence the extent of inhibition. SSA-dehydrogenase activity was not affected by HA-966 and DABA, while NH-DABA was only slightly inhibitory at 5.0 mM. However, the enzyme was strongly inhibited by zinc and by p-HBA. The concentration of 2-mercaptoethanol was very critical for the extent of inhibition observed for zinc. In the presence of 20 mM 2-mercaptoethanol a 20% inhibition

Table 2.6.3.

Rate of complex formation between PLP and drugs

Addition	Rate of complex formation ($mM^{-1}.min.^{-1}$)	(N)	Relative potency (%)	Not effective
Hydroxylamine	3.180 ± 0.402	(6)	100.0	GABA
Aminooxyacetic acid	0.812 ± 0.194	(3)	25.5	Morphine
Noradrenaline	0.510 ± 0.072	(3)	16.0	Adrenaline
NH-DABA	0.286 ± 0.066	(5)	9.0	DABA
Dopamine	0.276 ± 0.015	(3)	8.7	Apomorphine
L-DOPA	0.232 ± 0.021	(3)	7.3	Amphetamine
5-HT	0.009 ± 0.001	(2)	0.3	Tryptamine
5-HTP	0.009 ± 0.001	(2)	0.3	p-HBA
HA-966	0.008 ± 0.000	(2)	0.3	Glutamate
DPI	0.004	(1)		Zinc

Measurements were done at pH 6.5 in the presence of 0.4 mM of PLP. Reaction was started with the addition of the substances mentioned above and continued until the extinction at 385 nm did not decrease further. By plotting the ^{10}log of the decrease in extinction at 385 nm against time (see methods for details), straight lines were found, representing the rate of complex formation with PLP. From measurements at various drug concentrations the mean rate of complex formation / mM of drug was calculated (dimension: $min.^{-1}.mM^{-1}$). The following concentrations were used: Hydroxylamine: 0.81; 1.0 and 2.0 mM, Aminooxyacetic acid: 0.81; 2.0 and 5.0 mM, Noradrenaline: 1.0; 2.0 and 5.0 mM, 5-HT: 5.0 mM, 5-HTP: 5.0 mM, NH-DABA: 0.81; 2.9 and 8.1 mM, HA-966: 1mM; DPI: 5.0 mM.

was observed with 0.2 mM zinc, while a 67% inhibition of the enzyme was found with 5 mM 2-mercaptoethanol.

2.6.2. Complex formation with PLP

During the assay of glutamate decarboxylase activity in the presence of added PLP it was observed that the characteristic yellow colour of PLP disappeared during incubation with HA-966 and NH-DABA. Therefore, the interaction of these compounds with PLP was investigated spectrophotometrically under conditions approximating those of the glutamate decarboxylase activity assay. The rate of complex formation with PLP was estimated using semi-log plots of the decrease of the extinction at 385 nm with incubation time. In most cases linear

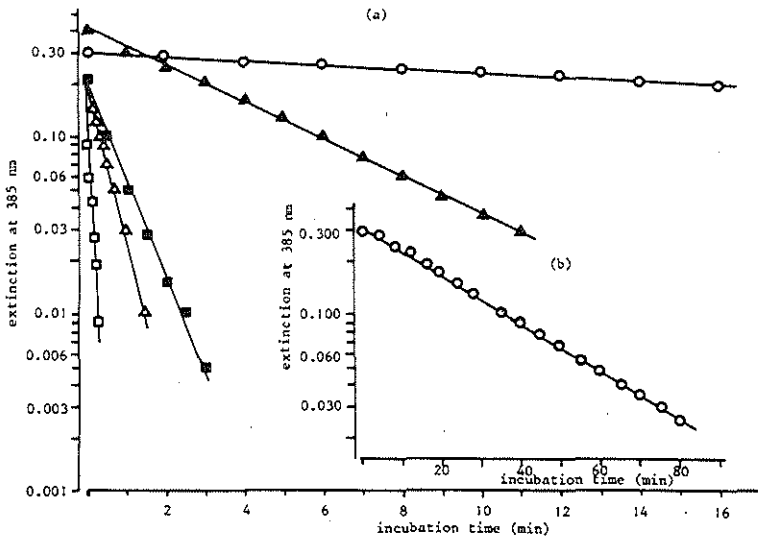


Fig. 2.6.1. a. The time course of complex formation with PLP for HA-966, NH-DABA and hydroxylamine. Measurements were done at pH 6.5 with 0.4 mM PLP. The reaction was started by the addition of the drugs and the time course of complex formation was followed until no further decrease of the extinction at 385 nm could be observed. The logarithm of the decrease of the extinction at 385 nm was plotted against time for: 8.0 mM HA-966 (–○–○–); 8.0 mM NH-DABA (–△–△–); 8.0 mM hydroxylamine (–□–□–); 0.8 mM NH-DABA (–▲–▲–) and 0.8 mM hydroxylamine (–■–■–).
b. The time course of complex formation with HA-966. The experimental details are as described under a; only the time scale is extended to show the full curve with 8.0 mM HA-966.

plots were found as demonstrated for HA-966, NH-DABA and hydroxylamine in Fig. 2.6.1. The results are summarized in Table 2.6.3 and show that hydroxylamine is most active. AOAA was about four times less active than hydroxylamine, NH-DABA was about ten times less active, while HA-966 was hardly effective at all. No complex formation could be observed for glutamate, GABA, DABA and some other compounds as shown in Table 2.6.3. In addition, many neurotransmitters and related substances were active complexing agents. The values for noradrenaline, dopamine and L-DOPA were somewhat underestimated because an initial increase of the yellow absorbance was observed during the first minute of incubation. This resulted in a deviation from linearity of the semi-log plot during the first minute. Similar results were obtained by HOLTZ & WESTERMAN (1957) for noradrenaline and L-DOPA.

2.6.3. Discussion

The differential effects of HAP, NH-DABA and DABA on glutamate decarboxylase and GABA-transaminase activity reported in the present study are probably related to the PLP-complexing properties of their 4-substituted amino-group. For example, NH-DABA is an effective inhibitor of these enzymes and it is also a potent complexing agent with PLP. The involvement of PLP in the effect of NH-DABA on glutamate decarboxylase activity is further shown by the decrease of the ratio of glutamate decarboxylase activity with and without added PLP, indicating that PLP partially restored glutamate decarboxylase activity. The parent pyrrolidone HA-966 was a weak inhibitor of glutamate decarboxylase in the absence of added PLP, while the rate of complex formation of HA-966 with PLP was comparatively low. The involvement of PLP in the inhibitory effect of HA-966 on the decarboxylase was indicated by the decrease of the ratio of glutamate decarboxylase activity with or without PLP. DABA was a weak inhibitor of glutamate decarboxylase and GABA-transaminase activity. It decreased the ratio of glutamate decarboxylase activity with or without PLP, but complex formation with PLP could not be demonstrated with DABA. These results confirm results of SHUTER & ROBINS (1974) with mouse brain GABA-transaminase and glutamate decarboxylase.
Some evidence for the inhibition of GABA-synthesis by HA-966 *in vivo* was provided by MÖHLER et al. (1975a and 1975b), who demonstrated that the conversion of the labelled precursor glucose into GABA as well as GABA-concentration were selectively diminished. The *in vitro* inhibition of glutamate decarboxylase activity by HA-966 via interference with its cofactor PLP might explain this phenomenon. However, *in vivo* conversion of HA-966 in its more active aliphatic analogue might also explain the observed inhibition of GABA formation *in vivo*.

Zinc has been implicated in the regulation of the GABA-system (Wu & ROBERTS, 1974): zinc is an important cofactor for PLP-kinase, it inhibits both glutamate decarboxylase and GABA-transaminase activity and its concentration in brain areas where presumed GABA-ergic synapses are concentrated is high (TWOMEY & BAXTER, 1973; WU & ROBERTS, 1974; SCHOUSBOE et al., 1974; FISHER & DAVIES, 1974; CRAWFORD & CONNER, 1972). The present study showed that glutamate decarboxylase and GABA-transaminase were both inhibited by zinc in agreement with these reports. In addition, the observed inhibition of SSA-dehydrogenase is another phenomenon demonstrating the importance of this metal ion for the regulation of the GABA system.

When using a coupled enzyme system for the measurement of GABA-transaminase activity the effect of the drugs under study on SSA-dehydrogenase has to be considered. Though both GABA-transaminase and SSA-dehydrogenase were inhibited by NH-DABA the effect on GABA-transaminase was much stronger. Since the effect of NH-DABA could be demonstrated with 0.5 mM NH-DABA, a concentration without effect on SSA-dehydrogenase, the inhibition of GABA-transaminase could not be ascribed to inhibition of the coupling enzyme. The same applies for the effect of zinc on GABA-transaminase. This enzyme was measured in the presence of 20 mM 2-mercaptoethanol. Under these conditions SSA-dehydrogenase was sufficiently active to convert all SSA formed into succinate. However, a slight inhibition of SSA-dehydrogenase during the coupled assay of GABA-transaminase might increase the concentration of intermediate SSA and might consequently increase the backward formation of GABA. It can not be excluded that such a mechanism contributed to the observed inhibition of GABA-transaminase by zinc or NH-DABA. The inhibition by p-HBA might be indirect since GABA-transaminase and SSA-dehydrogenase were inhibited to approximately the same extent. Again, this inhibition of SSA-dehydrogenase might activate the backward formation of GABA, resulting in an apparent inhibition of GABA-transaminase by p-HBA.

The inhibition studies with SSA-dehydrogenase indicate that the structural requirements for inhibition of the dehydrogenase differ from those observed for glutamate decarboxylase and GABA-transaminase. The most potent inhibitor of SSA-dehydrogenase, p-HBA, was without effect on glutamate decarboxylase, while the effect on GABA-transaminase must be investigated further because it may arise from inhibition of the dehydrogenase as discussed above. SSA-dehydrogenase was slightly inhibited by NH-DABA while DABA and HA-966 were without effect. On the other hand, glutamate decarboxylase and GABA-transaminase were strongly inhibited by NH-DABA, while both enzymes were also affected by DABA and glutamate decarboxylase was inhibited by HA-966. Therefore, these studies confirm the well known fact that a selective inhibition of glutamate decarboxylase or GABA-transaminase is difficult to achieve. However, the results demonstrate that selectivity might be obtained when SSA-

dehydrogenase inhibition is considered. Using p-HBA as an example, only GABA degradative enzymes were affected, while glutamate decarboxylase remained completely unaffected. This suggests that SSA-dehydrogenase inhibition by p-HBA *in vivo* might result in a selective inhibition of GABA degradation without effecting GABA synthesis via glutamate decarboxylase. Similarly, HA-966 might inhibit GABA synthesis selectively by interfering with PLP *in vivo*.

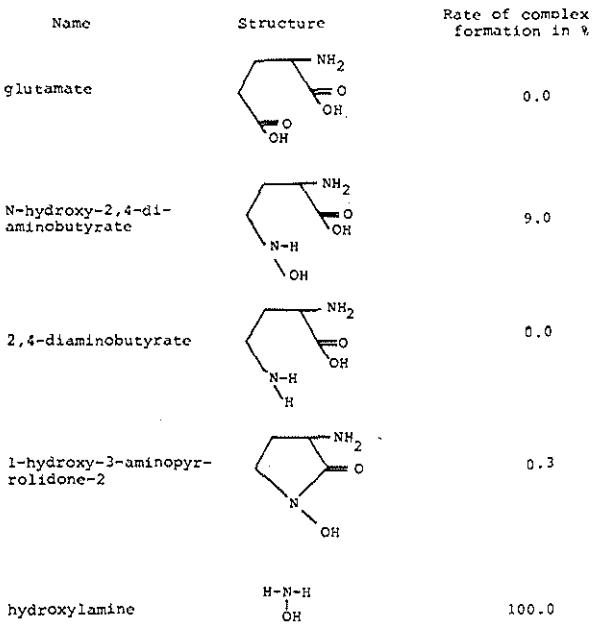


Fig. 2.6.2. Structure and rate of complex formation with PLP of some GABA analogues.

The experiments with PLP confirm the early observations of HOLTZ & WESTERMANN (1957) and experiments of WU & ROBERTS (1974) with catecholamines, demonstrating that many neurotransmitters and neurotransmitter precursors might form complexes with PLP, resulting in inhibition of glutamate decarboxylase activity as observed in the present study for HA-966 and NH-DABA. Since certain *in vivo* studies involve local application of L-DOPA, dopamine and noradrenaline in discrete brain areas and relatively large amounts are introduced, inhibition of glutamate decarboxylase activity via cofactor depletion should be considered as a possible factor in such studies. This is of special importance if the effect of the locally applied drug can only be observed

after a long latency (COSTALL et al., 1975a).
This study was initiated by the observation that zinc dyskinesias were antagonized by HA-966 (BONTA et al., 1971). As HA-966 and zinc have differential effects on the enzymes of the GABA-shunt *in vitro*, a role for the GABAergic system in the mediation of zinc-induced dyskinesias must be considered. The present results demonstrate that high concentrations of HA-966 might inhibit the synthesis of GABA *in vivo*. Zinc application to the brain may result in an increase of GABA, either by an increase of GABA formation via PLP-kinase activation or by a decrease of GABA degration as the result of inhibition of GABA-transaminase and SSA-dehydrogenase. However, the metal binding properties of HA-966 (BONTA et al., 1964) might also be responsible for the observed antagonism of zinc-induced dyskinesias (BONTA et al., 1971).
In conclusion, the present experiments indicate that PLP is important as the mediator of the inhibitory effect of NH-DABA, HAP and DABA on glutamate decarboxylase activity. The results suggest that the hydroxylamine-moiety is responsible for this inhibition. The efficacy of complex formation with PLP apparently depends on the number of substituents on the 4-N-nitrogen as demonstrated in Fig. 2.6.2. Though HA-966 is a relatively weak inhibitor of glutamate decarboxylase *in vivo* conversion into NH-DABA as suggested by BONTA et al. (1971) might strongly facilitate its inhibitory effect. Furthermore, the experiments with these GABA-like compounds suggest that a selective inhibition of GABA degradation might be obtained by inhibition of SSA-dehydrogenase as demonstrated here for p-HBA.

2.7. SELECTIVE AFFINITY FOR GABA-TRANSAMINASE OR SSA-DEHYDROGENASE: A CONCEPT FOR GABA-COMPARTMENTATION

No evidence has been presented demonstrating the storage of GABA in nerve fibers as has been described for other neurotransmitters like dopamine, noradrenaline, serotonine or acetylcholine. Accumulation of GABA in nerve terminals may occur for the following reasons. Synthesis may preferentially be located in nerve terminals, because very low glutamate decarboxylase activity has been demonstrated in glial elements (SALGANICOFF & DE ROBERTIS, 1963 and 1965; VAN KEMPEN et al., 1965; FONNUM, 1968; HAMBERGER & SELLSTRÖM, 1975; KURIYAMA, 1976; see also chapter 1.4.4). The suggestion that negligible GABA-transaminase activity is recovered has resulted in a model for the compartmentation of GABA in the CNS that is based on the differential localization of glutamate decarboxylase and GABA-transaminase activity in nerve endings and glial or neuronal elements respectively (VAN DEN BERG et al., 1975; see also chapter 1.2.9 and 1.2.10). However, the bulk of information favours the localization of a considerable portion of the GABA-catabolizing enzymes of the GABA-shunt in nerve endings (SALGANICOFF & DE ROBERTIS, 1963 and 1965; VAN KEMPEN et al., 1965; FONNUM, 1968; SIMS et al., 1971; SIMS & DAVIS, 1973; BUU & VAN GELDER, 1974; KATAOKA et al., 1974; HAMBERGER & SELLSTRÖM, 1975; see also chapter 1.4). The question can be put forward why GABA is accumulated in nerve endings, when a considerable portion of the GABA degrading enzymes are present in these nerve endings. The presence of GABA degrading enzymes would tend to metabolize GABA as soon as it is formed by the action of glutamate decarboxylase. One factor of considerable importance might be that an excess of glutamate decarboxylase activity is present in nerve endings when compared with GABA-transaminase and SSA-dehydrogenase activity. However, calculations based on lesion experiments of KATAOKA et al. (1974) demonstrate that such an excess of synthesizing capacity is not very likely (see chapter 1.4.5).

2.7.1. The backward reaction of GABA-transaminase

The kinetics of the GABA-transaminase reaction have been studied using purified enzyme (MAITRE et al., 1975; JOHN & FOWLER, 1976). The equilibrium constant as calculated from the individual rate constants for the forward and backward reaction of GABA-transaminase was 0.40 (JOHN & FOWLER, 1976), which is in reasonable agreement with the value of 0.29 observed by others (DUFFEY et al., 1972). This implies that under normal conditions of equilibrium the transamination reaction favours backward formation of GABA. Competitive inhibition of GABA and 2-oxoglutaric acid was observed for GABA-

transaminase with a K_i of 0.18 mM 2-oxoglutaric acid. The same phenomenon was observed for the backward reaction, SSA being a competitive antagonist of glutamate with a K_i of 0.66 mM.
Experiments with the radiochemical method to measure GABA-transaminase activity, originally meant to compare different methods (see chapter 2.3), revealed an interesting property of GABA-transaminase. The assay of GABA-transaminase using the radiochemical method was performed with and without NAD present in the incubation mixture. This means that in the presence of NAD SSA formed from GABA was further oxidized into succinate by the dehydrogenase reaction, while in the absence of NAD SSA was not further metabolized, resulting in accumulation of the intermediate SSA. It was expected that omission of NAD would have little effect on the amount of product (SSA alone or SSA + succinate) formed during the GABA-transaminase reaction. The results, summarized in Table 2.7.1, indicate that omitting the NAD results in inhibition

Table 2.7.1.

The effect of NAD on the GABA-transaminase reaction

Tissue conc. (mg/ml)	GABA-transaminase activity (μmol/g/h)		
	no NAD	0.9 mM NAD	ratio
2.22	9.96	30.64	0.325
6.67	11.46	29.81	0.384

GABA-transaminase activity was measured in a medium containing: 50 mM Tris-HCl, pH 8.5; 3.0 mM GABA; 2.0 mM 2-oxoglutaric acid; 20 mM 2-mercaptoethanol; 0.9 mM NAD and 2.22 or 6.67 mg tissue/ml; 1.0 mM succinate and 0.09 μCi (U-^{14}C)-GABA (spec. act. 4.6 mCi/mMol). GABA-transaminase activity was calculated from the conversion of ^{14}C-GABA in ^{14}C-SSA and/or ^{14}C-succinate as described in chapter 2.3. The incubation time was 30 min at 6.67 mg tissue/ml and 120 min at 2.22 mg tissue/ml. The results are the mean of two experiments.

of the rate of transamination. In the absence of NAD the rate of transamination of GABA in acidic products is about 30-40% of the control values in the presence of NAD. Under these conditions about 0.038 to 0.044 mM SSA was formed in the absence of NAD. In a second series of experiments time courses were measured under similar experimental conditions as described in the legend of Table 2.7.1, except that glutamate was added with a concentration of 1 mM. Under these conditions no difference was detected between the incubations in the presence or absence or absence of NAD. This suggests that glutamate decreased the backward formation of SSA into GABA.

2.7.2. Discussion

The only explanation of the sharp decrease of the net-formation of SSA and succinate from GABA, observed after omission of NAD, is that under these conditions part of the formed SSA will be converted into GABA. These results suggest that measurement of GABA-transaminase may give an underestimate of the true rate of transamination, if SSA is allowed to accumulate during the assay. This suggests that a reliable GABA-transaminase assay can only be obtained if the formed product or products (SSA and/or glutamate) are further converted. These results extend the observations in chapter 2.3, where ionic strength was suggested to explain much of the variability among methods used for the measurement of GABA-transaminase activity. The possibility that the observed accumulation of SSA may activate the backward formation of GABA from SSA has an other important bearing for the regulation of GABA-metabolism. It appears that the backward reaction is already 60-70% as active as the forward reaction in the presence of 0.04 mM SSA. Supposing that the V_m for both reactions (forward and backward reaction of GABA-transaminase) is similar these experiments indicate that the backward reaction is more than half-saturated by a concentration of 0.04 mM. This implicates that the K_m for GABA-transaminase of SSA is less than 0.04 mM. The experiments with varying SSA concentrations reported in chapter 2.3 demonstrated that the affinity of SSA for SSA-dehydrogenase might be high – a K_m of about 20 μM or even lower as reported by others (EMBREE & ALBERS, 1964; KAMMERAAT & VELDSTRA, 1968) – or low – a K_m of about 90 μM or more – depending on tissue concentration or other variables. Thus, SSA may have a preferential affinity for GABA-transaminase resulting in accumulation of GABA in some compartment where the affinity of SSA for SSA-dehydrogenase is low. Alternatively, SSA may have a preferential affinity for SSA-dehydrogenase resulting in oxidation of SSA into succinate in another brain compartment. In the former situation accumulation of GABA is fascilated as might be the case in the neuronal compartment where GABA is concentrated in nerve endings. In the latter situation no accumulation of GABA occurs and the dehydrogenase will immediately oxidize all SSA formed into succinate. This situation might occur in the glial compartment. This model for GABA compartmentation in the brain has the important implication that SSA-dehydrogenase is rate-limiting in the neuronal compartment, while GABA-transaminase is rate-limiting in the glial compartment.

An other important property of this model is that the selective affinity of SSA for GABA-transaminase over SSA-dehydrogenase is responsible for an increase of functionally available GABA. This suggests that GABA-accumulation may occur after administration of drugs that decrease the affinity of SSA for SSA-dehydrogenase while having no effect on the affinity of SSA for GABA-trans-

aminase. The observation that GABA accumulation occurs post-mortem because the NAD is quantitatively converted within seconds after death into NADH inhibiting SSA-dehydrogenase selectively by co-substrate depletion is directly comparable to the experiments observed here (PATEL et al., 1974). Under these conditions GABA concentration doubled within a few minutes after death, although it is not clear where the accumulation of GABA was located. The experiments with 1.0 mM glutamate include the level of glutamate in the regulation of GABA degradation.

3. BEHAVIOURAL SECTION

3.1. INTRODUCTORY EXPERIMENTS

"A major contributing factor to rapid advances in understanding the functional role of CNS neurotransmitters is frequently the development of model systems which reflect the synaptic activity of these transmitters. Such models are valuable in their own right in that they may indicate some of the major physiological or behavioural processes in which the transmitter is involved. More importantly, however, these functional models are useful for examining the parameters of synaptic function, the specific action of drugs presumed to affect a transmitter system, and for the development of new drugs with desired actions."

Barry L. Jacobs, 1976

The starting point of the behavioural experiments were studies with HA-966 for which an action via the extrapyramidal GABA-ergic system was proposed because of its structural similarity with the GABA analogue γ-butyrolacton (BONTA et al., 1971; BONTA, 1973). Experiments with mice indicated that HA-966 antagonized a zinc-induced syndrome, consisting of hind limb tremor, running fits, disturbed gait and backward movement (BONTA et al., 1964 and 1971). Experiments with labelled amino acid precursors suggested that HA-966 selectively affected the flux of glucose towards amino acids and it was suggested that the communication between the metabolic compartments, mediated via glutamine and GABA (see chapter 1.2.9), was reduced (MÖHLER et al., 1975a and 1975b). As shown in chapter 2.6, HA-966 inhibited glutamate decarboxylase via complex formation with PLP, while it has been shown that all three GABA shunt enzymes are inhibited by zinc (WU & ROBERTS, 1974; SCHOUSBOE et al., 1974; FISHER & DAVIS, 1974; this thesis, chapter 2.6). It was concluded that the zinc induced syndrome and the inhibition of this syndrome by HA-966 might be caused by the differential effects of zinc and HA-966 on the GABA-shunt enzymes.
IZUMI et al. (1973) have described a yawning-stretching syndrome which could be evoked by the intraventricular injection of zinc chloride. Since the biochemical experiments indicated that zinc might interfere with GABA synthesis and degradation, this yawning-stretching behaviour might be related to changes in the GABA-ergic system. Therefore, the experiments with zinc were repeated, while NH-DABA, having a similar inhibition pattern on the GABA shunt enzymes (see chapter 2.6), was also included. The purpose of these experiments was to see whether this yawning-stretching syndrome could be used as a be-

haviour al correlate of increased or decreased GABA-ergic activity in the brain. It must be emphasized that neither drug acts selectively, since both GABA synthesis and GABA degradation were affected.

3.1.1. Experimental

Male Wistar random rats (200 g of weight) were implanted with stainless steel canules under Hypnorm® narcose near the lateral ventricle. After a recovery period of 7 to 10 days the animals were used for the *in vivo* experiments. One group of rats was injected with zinc or NH-DABA with a microsyringe placed in the implanted guiding cannule penetrating into the lateral ventricle. The rats were placed in a stainless steel cage without saw dust on the bottom and observed for 2 h after injection. Another group of rats with similarly implanted guiding cannules were injected without restraint using a syringe connected via a polyethylene tubing on an injection needle penetrating the lateral ventricle. The behaviour of the rats was observed for 60 min in plastic transparant cages with saw dust on the bottom using scoring systems for yawning-stretching (IZUMI et al., 1973), GABA-induced dyskinesias (McKENZIE et al., 1975), dopamine related oral-buccal stereotyped dyskinesias (COSTALL & NAYLOR, 1975a) and morphine abstinence (BLÄSIG et al., 1973; COLLIER & FRANCIS, 1975; HINE et al., 1975; WATANABA, 1971; WEI, 1973).

3.1.2. Effect of zinc and NH-DABA *in vivo*

In some rats yawning-stretching was indeed observed for a period of 10 min with a frequency of 10 to 20 times per 2 h observation period as described by IZUMI

Table 3.1.1.

Comparison between quasi-morphine abstinence behaviour and the effects of zinc chloride and NH-DABA

jumping (+?)	diarrhoea (–)	rearing
teeth chatter (+)	salivation (–)	restlessness
irritation when touched (+)		body shakes
irritation when handled (+)		
chewing (+)		
ptosis (+)		
head shakes (+)		
paw tremor (+)		
ejaculation/penis licking (+)		

(+): observed; (–): absent; no indication: no special attention was paid to it.

et al. (1973). However, other symptoms including hind-limb tremors, irritation when handled, hunch-back posture, teeth chatter, grimacing and penis licking were also observed. In general, initial increased activity with jumping for 5 min with 8-12 μg zinc chloride as an extreme, was followed by a period in which the rat was not particularly active. During this period dyskinesiasis and yawning-stretching occurred, while irritation developed especially during the second day post-treatment. The observed behaviour appeared to be markedly similar to a list of symptoms described by COLLIER et al. (1972), to characterize quasi-mor-

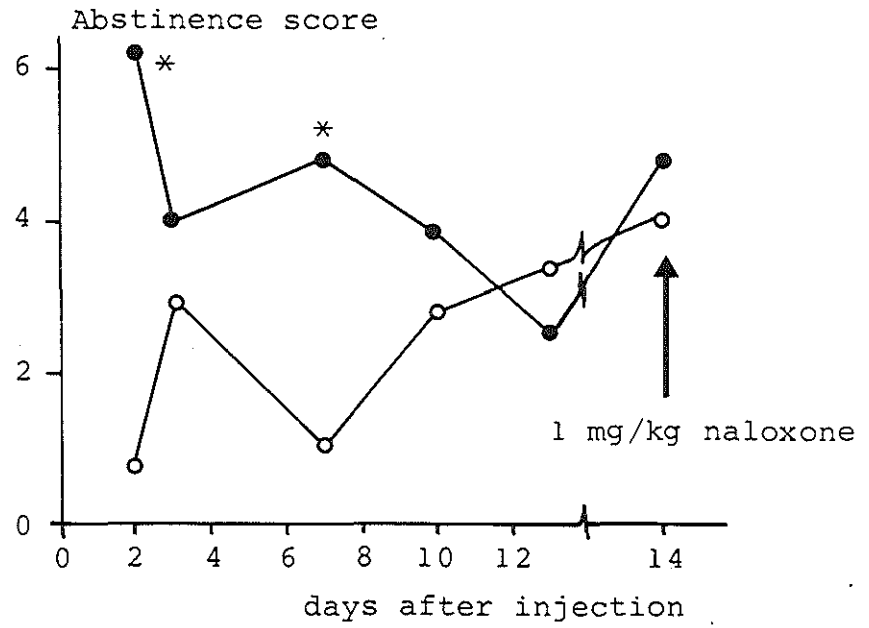


Fig. 3.1.1. Abstinence scores after treatment with Hypnorm®.
A group of 10 rats received saline (–○–○–) and another group of 15 rats (–●–●–) received 0.15 ml Hypnorm®. Abstinence was assessed using the method of FREDERICKSON & SMITH (1973), see methods. *: $p < 0.001$, Mann Whitney U-test, one tailed.

phine abstinence behaviour after phosphodiesterase inhibition (Table 3.1.1).
A further group of 6 rats was injected with 1, 2 or 4 μg zinc (as zincsulphate), 1 or 2 μg NH-DABA, or saline. A second series of experiments was performed two days after the first series. These experiments were discontinued after two sessions because long-term effects were observed with the treatments. Moreover, the treatment with Hypnorm® (containing 10 mg/ml of the neuroleptic fluanisone and 0.2 mg/ml of the narcotic analgesic fentanyl) might interfere with the

behaviour observed after zinc or NH-DABA administration. A detailed analysis of the results for individual signs revealed no significant difference between the various treatments and the saline treatment. However, a very pronounced weightloss was observed with zinc and NH-DABA. With 4 μg zinc a weightloss of 33 and 16 g was observed, while the weightloss with 2 μg NH-DABA was 7 and 17 g. Saline treated rats lost 2 and 4 g.

3.1.2. Hypnorm® induced abstinence

From a group of 25 rats 15 were treated with 0.15 ml Hypnorm® while 10 were treated with saline. Body weight and abstinence scores were followed for 14 days. At day 14 all rats received 1 mg/kg naloxone to see whether abstinence behaviour could be precipitated. Although initially abstinence scores after treatment with Hypnorm® were significantly different from the control group, no significant effect was detectable after 14 days after treatment with 1 mg/kg naloxone.

3.1.3. Discussion

The aim of the experiments described in this chapter was to study whether the zinc-syndrome (BONTA et al., 1964 and 1971; IZUMI et al., 1973), which was related to the GABA-ergic system by the *in vitro* experiments (see chapter 2.6), could be useful as a behavioural correlate of increased GABA-ergic activity. Because zinc lacked specificity in the biochemical experiments, the possibility of an inhibition of GABA-ergic activity is also possible. The experiments with zinc and NH-DABA failed to produce consistantly yawning-stretching. However, a number of symptoms were observed that were similar to the ones observed by COLLIER et al. (1972) and classified as quasi-morphine abstinence behaviour because similar signs were observed during morphine abstinence. A second series of experiments with zinc and NH-DABA gave significant scores for abstinence, but saline was equally effective. The effects on body weight were more consistant and dose-related. Since weight loss has been classified as a very prominant and reproducible abstinence sign and no substantial effect was observed after saline treatment, there may be some relationship with morphine abstinence (WEI, 1973). However, the observation of abstinence behaviour after one dose of Hypnorm® as was used during operation of the rat, suggests that hypnorm might interfere with the behavioural signs after zinc or NH-DABA administration.

In conclusion, these preliminary experiments indicate that inhibition of GABA-degradation may result in the development of a syndrome classified as quasi-

morphine abstinence behaviour. Until sofar no experiments with inhibitors of GABA synthesis or GABA-degradation have shown that abstinence behaviour could arise after modifying the GABA-ergic system. In fact, inhibition of GABA synthesis results in convulsions (TAPIA, 1975; WOOD, 1975) while inhibition of GABA-transaminase results in sedation as has been observed after AOAA or ethanolamine-O-sulphate. The finding of HARVEY et al. (1975) that DPA was an inhibitor of SSA-dehydrogenase and lacked any effect on GABA-transaminase prompted us to study the effect of this compound. It is important in this respect that both zinc and NH-DABA are inhibitors of SSA-dehydrogenase *in vitro*. Because inhibitors of GABA-transaminase produce sedation it is not very likely that the effect of zinc and NH-DABA was caused by the inhibition of GABA-transaminase. Therefore it seemed that inhibition of SSA-dehydrogenase was the common underlying phenomenon connecting the effects of zinc and NH-DABA. Since DPA has a selective effect on SSA-dehydrogenase and because it can be administered intraperitoneally, further studies were conducted with this drug.

3.2. MEASUREMENT OF MORPHINE- AND GABA-INDUCED ABSTINENCE BEHAVIOUR IN THE RAT

Physical dependence indicates that a normal functioning of the organism is only possible in the presence of certain amounts of opiate. Withdrawal of the opiate, either by stopping the chronic treatment or by injecting a morphine antagonist like naloxone, will result in an abstinence syndrome. The severity of this abstinence syndrome is used to measure the degree of physical dependence (see for more details, chapters 1.6.1 to 1.6.4).
Experiments described in chapter 3.1 indicated that inhibition of GABA metabolism resulted in a behavioural syndrome with many signs in common with the morphine abstinence syndrome (COLLIER et al., 1972). Morphine abstinence behaviour has been distinguished in central signs and autonomic signs (FREDERICKSON & SMITS, 1973; JHAMANDAS et al., 1973; FREDERICKSON, 1975). The behavioural syndrome probably provoked by inhibition of GABA degradation was characterized by central signs, whereas autonomic signs were never observed. Moreover, the method described by FREDERICKSON & SMITS (1973) seemed very proper to detect and quantify weak abstinence behaviour. Therefore, this method was selected to characterize GABA-induced abstinence behaviour.
In this chapter morphine abstinence will be compared with GABA-induced abstinence behaviour. Furthermore, the method of FREDERICKSON & SMITS (1973) for the measurement of abstinence behaviour will be described.

3.2.1. Materials and methods used for the characterization of DPA-induced abstinence behaviour

Animals: The experiments were performed with male Wistar random rats (TNO, Zeist, The Netherlands) weighing between 100 and 200 g. The animals were housed in a plastic transparant cage with food and water *ad libitum*. Lights were on from 7 a.m. to 9 p.m. The experiments were performed between 9 a.m. and 3 p.m. in a room with white noise and a constant temperature of 22°C.
Abstinence behaviour: At the start of an experiment the animal was placed in a plastic transparant cage (base area: 47 x 27 cm; height: 15 cm) with sawdust on the bottom. After a 30 min habituation period DPA was injected intraperitoneally and the behaviour was observed during a period of 15 min by an observer who did not know which treatment was given to the animal. Abstinence was assessed using the scoring method of FREDERICKSON & SMITS (1973). Scores of 0 (absent), 2 (mild) or 4 (marked) were given for salivation, rhinorrhea, lacrimation, urination, diarrhoea, erection, ejaculation, ptosis, teeth chatter, swallowing, tremor, hunchback posture, piloerection, irritability to handling, and

reaction to poking. Escape behaviour (i.e. only digging under our experimental conditions), body shaking, head shaking, foreleg shaking and yawning were counted as quantal events and assigned scores of 2, 4, 6 or 8 if they occurred 2-5, 5-10, 10-20 or more than 20 times, respectively, during the observation period of 15 min or during subperiods of 5 min. The sum of all scores (indicated in the figures as the mean score per animal ± S.E.) provided the total abstinence score. A summary of the method is given in Table 3.2.1, where also is indicated which signs are observed using DPA.

Table 3.2.1.

Points attributed to the scored abstinence signs according to Frederickson & Smits

1. Signs are scored 0 (absent), 2 (mild) and 4 (marked):

Salivation	Penal erection*	Tremor*
Rhinorrhea	Ejaculation	Hunchback posture*
Lacrimation	Ptosis*	Piloerection*
Urination	Teeth chatter*	Irritation to handling
Diarrhea	Swallowing*	Irritation to poking

2. Counted signs are scored 2, 4, 6, 8, if they occurred 2-5, 5-10, 10-20 or more than 20 times respectively.

Escape digging*	Body shaking*	Foreleg shaking*
Escape jumping	Head shaking*	Yawning

Scoring method according to FREDERICKSON & SMIT, 1973.
*: Observed after DPA administration, 300 mg/kg (see further)

Animal activity measurements: Animal activity was continuously monitored during observation of the animal using a Varimex activity meter (model B-1-75-1), equiped for alternating measurement of horizontal or vertical activity separately every 10 sec. Horizontal activity (H), vertical activity (V), total activity (H + V) and the ratio of horizontal to vertical activity (H/V-ratio) were separately estimated for each experiment. Vertical activity mainly consisted of rearing and grooming. No jumping occurred under the used experimental conditions.
Catalepsy: At the end of each experiment 15 min after the injection of DPA the rats were tested for the presence of catalepsy by placing the rat by its forepaws on a cork of 7 cm height. The rats were considered to be cataleptic if they remained longer than 15 sec in that position.
Righting reflex: The presence of a normal righting reflex was tested by placing the rats on their back. If they remained for 5 sec in that position the righting reflex was considered to be absent.
Scoring-sheet: A copy of the scoring sheet used for the behavioural experiments is shown in Fig. 3.2.1. This scoring sheet has been used with only minor changes for all experiments except those described in chapter 3.1.

SCORING ABSTINENCE USING THE SYSTEM OF FREDERICKSON AND SMITH.

ITEM	0 - 5 min	5 - 10 min	10 - 15 min	0 - 15 min
SALIVATION				
RHINORRHEA				
LACRIMATION				
URINATION				
DIARRHEA				
ERECTION				
EJACULATION				
PTOSIS				
TEETH CHATTER				
SWALLOWING				
TREMOR				
HUNCHBACK POSTURE				
PILOERECTION				
IRRITATION HANDL.				
REACTION POKING				

ESCAPE DIGGING				
ESCAPE JUMPING				
WET DOG SHAKES				
HEAD SHAKES				
FORELEG SHAKES				
YAWNING				
TOTAL SCORE				

MOTOR ACTIVITY	0 - 5 min	5 - 10 min	10 - 15 min	0 - 15 min
HORIZONTAL ACTIVITY				
VERTICAL ACTIVITY				
TOTAL ACTIVITY				
HOR./ VERT. RATIO				

ANIMAL	
TREATMENT	
WEIGHT	
DATE	
GENERAL REMARKS	

EXPLORATORY ACTIVITY	
CATALEPSY (TIME AND SCORE)	
CONVULSIONS (TIME AND INCIDENCE)	
RIGHTING REFLEX	

Fig. 3.2.1. Scoring-sheet used for measuring DPA-induced abstinence behaviour.

3.2.2. Morphine abstinence induced by a single, high dose of morphine

To compare the scores obtained in DPA treated rats with those obtained after morphine treatment, rats were challenged with the high dose of 150 mg/kg morphine. After 75 min 1, 4 or 10 mg/kg naloxone was given. A second group was pretreated with saline or naloxone as described above. The results in Fig. 3.2.3 show that scores up to 16 could be obtained with this method, although relatively high amounts of naloxone were required to obtain a maximum effect. Comparable scores are also found after DPA treatment as will be described in the forthcoming chapters.

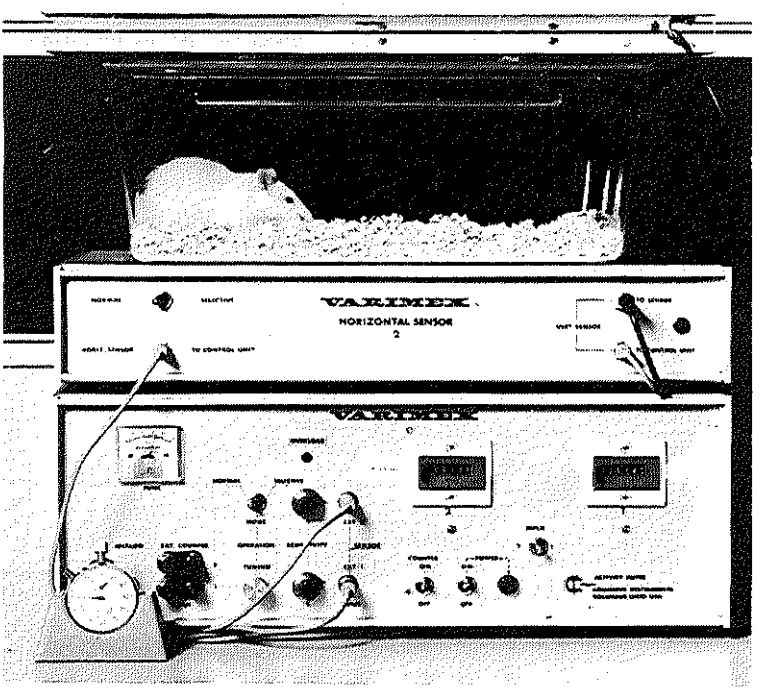


Fig. 3.2.2. The Varimex activity meter used for the behavioural experiments.

3.2.3. Comparison of morphine- and DPA-induced abstinence behaviour

A comparison of abstinence scores as reported in the literature for morphine abstinence with those obtained for DPA-induced abstinence was made. Since the scoring system as used by FREDERICKSON & SMITS (1973) was used

present study, a comparison with their scores might give some indication for the relevance of the severity of the observed DPA-induced abstinence behaviour in relation to morphine abstinence. However, two factors must be considered.

Table 3.2.2.

Abstince scores in different strains of rats.

Strain	abstinence score	percentage
Sprague Dawley	36	100
Holtzman	28	80
Wistar	25	70

A multiple injection schedule was used to make the rats dependent (from JHAMANDAS et al., 1973).

JHAMANDAS et al. (1973) observed marked differences in sensitivity for morphine abstinence in different strains of rats. He reported that the Wistar rat, which was used for the present studies, was 30% less sensitive than the Sprague Dawley rat. Besides this lower sensitivity to morphine, the DPA-induced abstinence signs only represented the central signs of morphine abstinence,

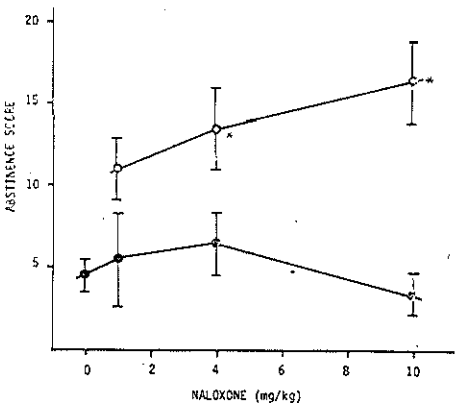


Fig. 3.2.3. Precipitated morphine abstinence after a single dose of morphine. Rats were pretreated with 150 mg/kg morphine (intraperitoneally) or saline and received saline, 1, 4 or 10 mg/kg naloxone after 75 min. The behaviour was observed for 15 min and abstinence was assessed using the scoring method of FREDERICKSON & SMITS (1973). Abstinence scores refer to the mean score per animal ± S.E. Significance was tested using the Mann-Whitney U-test, one tailed. Every experimental point is the mean of 4 rats.
*: $p < 0.05$; **: $p < 0.002$, when compared with the treatment with 4 or 10 mg/kg naloxone alone.

whereas both central and autonomic signs contributed to the morphine abstinence scores as measured by FREDERICKSON & SMITS (1973). If a correction is made for both factors it appeared that the DPA-induced abstinence behaviour was less severe than morphine abstinence behaviour measured at peaking time.

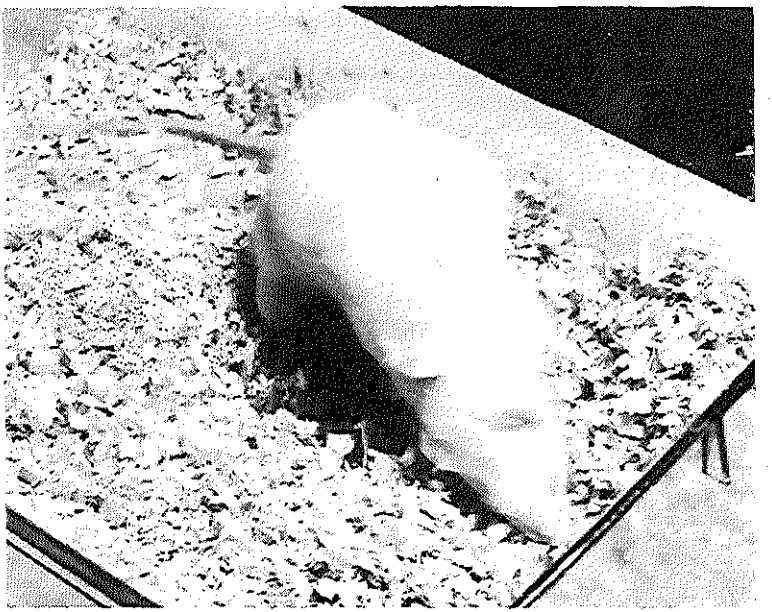


Fig. 3.2.4. Shaking behaviour of the rat.

Table 3.2.3.

Different types of abstinence in morphine dependent rats.

	abstinence scores	percentages
a. Total signs	31 ± 2	100
Central signs	25 ± 2	80
Autonomic signs	7 ± 1	20
b. Peak score 48 hrs after pre-treatment with a sustained release prep.		
Total signs	54 ± 4	

Abstinence scores were measured with a slightly modified system under a. according to FREDERICKSON (1975). Under b. abstinence behaviour was exactly measured as described in the present study according to FREDERICKSON & SMITS (1973). These results were taken from FREDERICKSON (1975) (a) and FREDERICKSON & SMITS (1973) (b).

3.2.4. Discussion

The results in Fig. 3.2.5 and Tables 3.2.2, 3.2.3 and 3.2.4 demonstrate that, after correction for strain differences and the contribution of autonomic signs to morphine abstinence scores, the DPA-induced abstinence is considerably

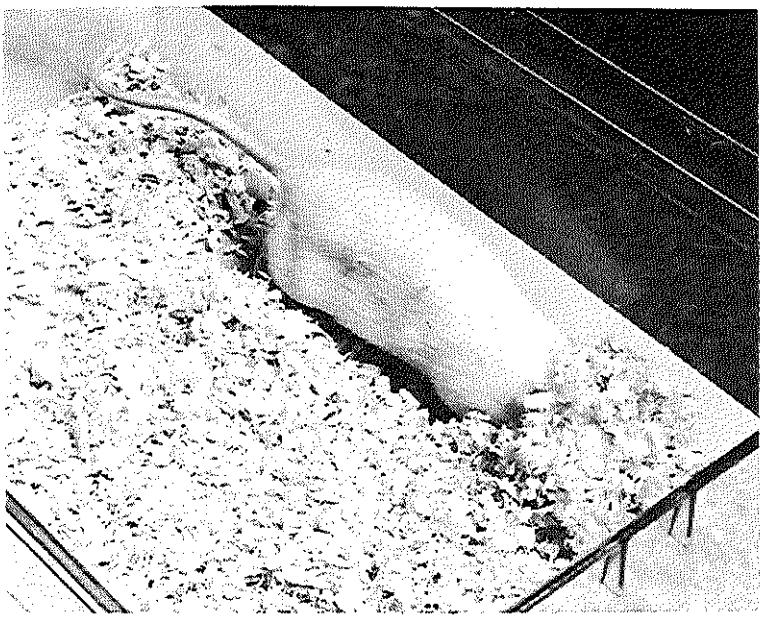


Fig. 3.2.5. Escape digging of the rat.

Table 3.2.4.

Comparison of abstinence scores using different models.

Corrected* peak values obtained from FREDERICKSON & SMITS (1973):	30 ± 2
Peak values obtained with 300 mg/kg DPA (present study):	20 ± 2
Peak values obtained with 150 mg/kg morphine (Fig. 3.2.5.):	16 ± 2

*: A correction was made for strain differences and the contribution of autonomic signs, see also chapter 3.3.

weaker than the morphine abstinence syndrome as measured by FREDERICKSON & SMITS (1973). However, such a comparison ignores the fact that two different observers have done these experiments. A comparison of the abstinence behaviour observed after treatment with morphine with that after administration of DPA is allowed. The results demonstrate that DPA-induced

abstinence behaviour results in higher scores than this special type of morphine abstinence. These studies suggest that DPA-induced abstinence behaviour has a severity which is somewhere intermediate between single-dose abstinence and the abstinence syndrome obtained with the pellet method. However, a further comparison using the pellet method and a multi-injection schedule seems worthwhile.

3.3. SUPPRESSION OF GABA-INDUCED ABSTINENCE BEHAVIOUR IN NAIVE RATS BY MORPHINE AND BICUCULLINE

Precipitated abstinence refers to an abstinence syndrome produced by a morphine antagonist in physically dependent animals, which cannot be attributed to a combination of the independent actions of the narcotic and the narcotic antagonist (MARTIN, 1967). By simultaneous assessment of morphine tolerance and physical dependence it is demonstrated that a close relationship exists between the two phenomena, suggesting that a common underlying mechanism might be involved (WAY et al., 1969). Recently, COLLIER and coworkers (COLLIER et al., 1974; FRANCIS et al., 1975) have shown that in rats which had not been previously subjected to opioid treatment abstinence behaviour could be induced by injecting theophylline, a phosphodiesterase inhibitor. We were interested in finding a behavioural correlate of increased GABA-ergic activity in the CNS. Initial experiments indicated that intraventricular injection of drugs that inhibited GABA metabolism provoked a behavioural syndrome with many signs in common with the morphine abstinence syndrome. To allow peripheral administration of the drugs we have studied the behavioural effect of n-di-propylacetate (DPA), an anti-petit mal drug known to inhibit GABA-metabolism in the brain (SIMLER et al., 1973; HARVEY et al., 1975). DPA administration to rats or mice causes an increase of GABA concentration in the brain with a maximum being reached in about 30 to 45 min (GODIN et al., 1969; CIESIELSKI et al., 1975).

3.3.1. Experimental

The experiments were performed with male albino Wistar random rats weighing 125 to 150 g. DPA was administered in a volume of 3 ml/kg, while morphine, naloxone and bicuculline were administered in a volume of 1 ml/kg. Abstinence behaviour, activity parameters, catalepsy and convulsions were noted. In this chapter the results for abstinence behaviour and motor activity parameters will be discussed, while some other aspects including catalepsy and convulsions with bicuculline are discussed in chapters 3.9 and 3.10. Further experimental details are described in the legends to the figures.

3.3.2. Effect of graded doses of DPA on abstinence behaviour

Intraperitoneal administration of DPA to the rat produced a behaviour with many characteristics of the morphine abstinence syndrome (WEI et al., 1973; BLÄSIG et al., 1973; COLLIER et al., 1973), including shaking behaviour,

escape digging, hunchback posture, piloerection and ptosis, while penis licking and swallowing were less frequently observed. The DPA-induced abstinence behaviour started within 2 to 3 min after injection, reached peak values between

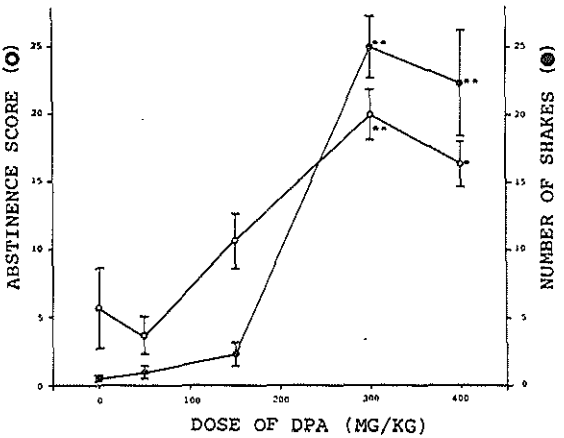


Fig. 3.3.1. Effect of graded doses of DPA on abstinence behaviour and number of body shakes. The rats were observed for 15 min, 6 rats per dose. Abstinence score and number of shakes refer to the mean score per animal ± S.E. Statistics (Mann Whitney U test):
*: $p < 0.002$; *: $p < 0.001$ when compared with the saline treated controls.

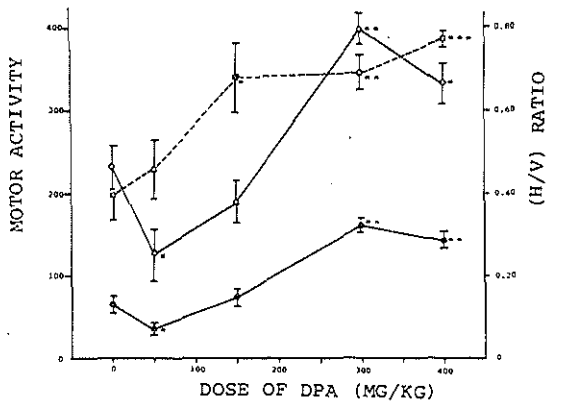


Fig. 3.3.2. Effect of graded doses of DPA on motor activity parameters. The experiments were performed as described in the legend of Fig. 3.3.1. Motor activity parameters refer to the mean score per animal ± S.E. Statistics (Student's t-test):
*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ when compared with the saline treated group.

5 and 10 min after DPA administration and was not present after 15 min. The results shown in Fig. 3.3.1 demonstrate that the administration of graded doses of DPA induced abstinence behaviour in a dose dependent manner. When the frequency of shaking behaviour was plotted against the dose of DPA a similar dose-response curve was obtained. Since maximal scores were obtained with 300 mg/kg DPA, all further studies were performed with this dose.

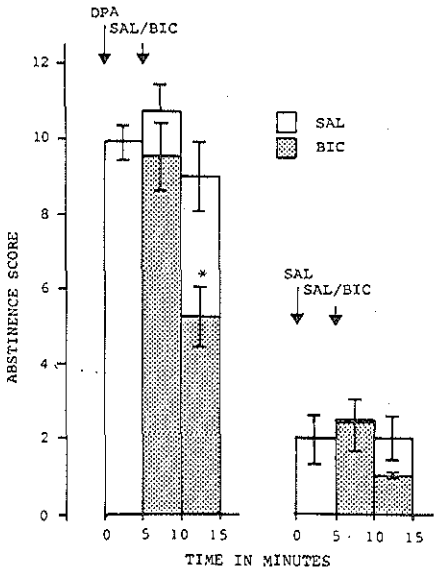


Fig. 3.3.3. The effect of bicuculline on DPA induced abstinence behaviour.
A. Two groups of 16 rats were pretreated with 300 mg/kg DPA at zero time. After 5 min one group was injected with 2 mg/kg bicuculline, dissolved in saline at pH 3.5 (BIC) and the other group received saline with a similar pH (SAL). The severity of abstinence behaviour was assessed for the three 5 min periods separately and is given as the mean score per animal ± S.E. Three convulsing animals in the bicuculline treated group were omitted from the results.
B. Two groups of 4 rats were pretreated with saline (SAL) at zero time. After 5 min one group was treated with 2 mg/kg bicuculline (BIC) while the other group received saline (SAL). Abstinence was assessed as described under A. Statistics (Mann Whitney U-test, one tailed):
*: $p < 0.01$ when compared with the saline treated rats.

3.3.3. Effect of graded doses of DPA on animal activity

DPA administration increased the activity of the animal considerably above control levels of saline treated rats (Fig. 3.3.2). However, at 50 mg/kg DPA a

significant depression of total and horizontal activity was observed. Though both horizontal and vertical activity increased with the dose, a significant increase of the H/V ratio was observed at 150, 300 and 400 mg/kg DPA.

3.3.4. Effect of the GABA antagonist bicuculline

To study whether the DPA-induced abstinence behaviour was the result of an action of GABA at its receptor sites the GABA antagonist bicuculline was used. Pretreatment of the rats with bicuculline did not prevent the DPA-induced abstinence behaviour. However, the behaviour could significantly be suppressed by the subconvulsive dose of 2 mg/kg bicuculline injected 5 min after DPA treatment. As shown in Fig. 3.3.3, this treatment suppressed the behavioural syndrome during the observation period from 10 to 15 min. Also the H/V ratio decreased from 0.785 ± 0.055 (16) for the saline treated group to 0.622 ± 0.090 (13) for the bicuculline treated group during this observation period (Mann Whitney U-test, $p < 0.02$). This decrease in H/V ratio is caused by a decrease in horizontal activity from 82.3 ± 7.7 (16) in saline treated controls to 61.2 ± 10.8 in the bicuculline treated group (Mann Whitney U-test, one tailed: $p < 0.05$), while vertical activity was not affected.

3.3.5. The effect of morphine on DPA-induced abstinence behaviour

To evaluate whether the DPA-induced abstinence behaviour can be considered as "quasi-morphine abstinence behaviour" the effect of morphine on DPA-induced abstinence behaviour was studied. The results in Fig. 3.3.4 show that a low dose of 1 mg/kg morphine is able to suppress the effects of DPA. Subsequent administration of naloxone (1 mg/kg) could release the abstinence behaviour in the morphine treated group, but was without effect in rats receiving saline instead of morphine.

3.3.6. The effect of morphine on motor activity parameters

Administration of DPA increased motor activity considerably above levels obtained in saline treated rats (Table 3.3.1a). As shown in Table 3.3.1b injection of morphine in a dose of 1 mg/kg (i.p.) to DPA pretreated rats reduced this increase in activity to a level comparable to the activity measured in saline treated controls. Also the H/V ratio was significantly reduced by morphine from 0.898 ± 0.057 before to 0.648 ± 0.091 after its administration ($n = 11, p < 0.05$, Mann Whitney U-test, one tailed), while saline treatment was without effect on the

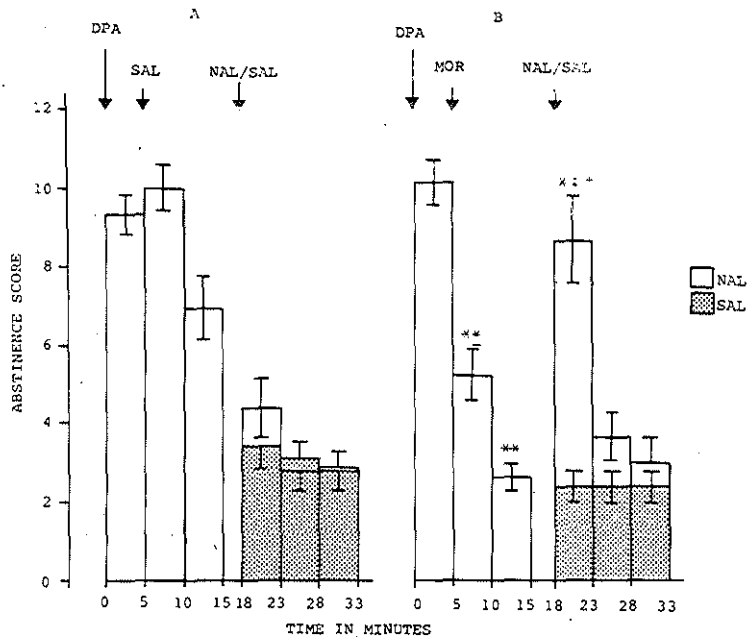


Fig. 3.3.4. The effect of morphine (MOR) and naloxone (NAL) on DPA induced abstinence behaviour. Four groups of 6 rats received 300 mg/kg DPA at the start of the experiment. After 5 min two groups were treated with 1 mg/kg morphine, while the other two groups received saline (SAL). After 18 min 1 mg/kg naloxone was administered to one morphine and to one saline treated group, while the remaining two groups received saline. Therefore, four groups were considered: DPA x MOR x SAL; DPA x MOR x NAL; DPA x SAL x SAL and DPA x SAL x NAL. The saline treated groups are presented under A, while the morphine treated groups are presented under B. Since in these experiments the modulatory effect of morphine on DPA induced abstinence behaviour was investigated 2 out of 24 rats showing no abstinence behaviour during the first 5 min (having a score of 2 or less) were omitted. All bars refer to the mean score per animal ± S.E. Statistics (Mann Whitney U-test, one tailed):
†: $p < 0.005$ when compared with DPA x SAL x NAL controls. *: $p < 0.05$ when compared with DPA x SAL x NAL controls; **: $p < 0.001$ when compared with DPA x SAL controls.

H/V ratio. However, increasing the dose of morphine to 2 mg/kg resulted in a decrease in motor activity to 20% of that obtained in saline treated controls.

Table 3.3.1.

The effect of morphine on motor activity parameters after DPA treatment

Treatment (mg/kg) after			Total activity / 5 min period		
0 min	5 min	N	0-5 min	5-10 min	10-15 min
a. DPA	saline	8	235.6±33.3*	218.9±20.8*	178.8± 7.5*
saline	saline	9	82.7±18.8	63.0±18.0	37.8±24.2
b. DPA	saline	12	226.7±13.3	202.8±17.6	162.2±17.3
DPA	morphine (1)	12	232.0±14.6	75.7±19.6*	37.6±24.3*
DPA	morphine (2)	12	228.6±17.0	34.9±10.4*	7.1± 3.8*

a. Two group of rats were treated with saline 5 min after administration of DPA or saline. Total activity was measured as the sum of horizontal and vertical activity and is indicated as the mean ± S.E.

b. Three groups of 12 rats were pretreated with 300 mg/kg DPA and received 1 or 2 mg/kg morphine or saline after 5 min. The first two groups (with saline and with 1 mg/kg morphine) refer to the experiment presented in Fig. 3.3.4., where further details are described.

Statistics (Student's t-test): *: $p < 0.005$, when compared with SAL x SAL (a) or DPA x SAL (b).

3.3.7. The effect of naloxone on DPA-induced abstinence behaviour

The specificity of the suppression by morphine of DPA-induced abstinence behaviour and its counteraction by naloxone was further shown by performing the same experiment as described above, but now injecting morphine (1 mg/kg) and naloxone (1 mg/kg) simultaneously. The results in Fig. 3.3.5 show that in DPA pretreated rats the administration of a mixture of morphine and naloxone was without effect when compared with the saline treated group. Furthermore, it can be concluded from Fig. 3.3.5b that the administration of naloxone alone did not modify abstinence behaviour. In addition, the effects of morphine on motor activity parameters did not occur after simultaneous treatment with morphine and naloxone.

3.3.8. Discussion

The functional activity of the GABA-ergic system has been investigated using seizure induction or seizure inhibition by inhibition of glutamate decarboxylase or GABA-transaminase activity respectively (TAPIA, 1975). Only recently other behavioural parameters like operant behaviour have been associated with changes in the functional activity of the GABA system (KENT & FEDINETS, 1976).

The present study demonstrates that increased GABA-ergic activity, obtained by intraperitoneal administration of DPA, is associated with a behavioural syndrome which resembles the morphine abstinence syndrome.
DPA is known to inhibit GABA metabolism selectively leaving GABA synthesis relatively unimpaired (GODIN et al., 1969). Although it was originally claimed

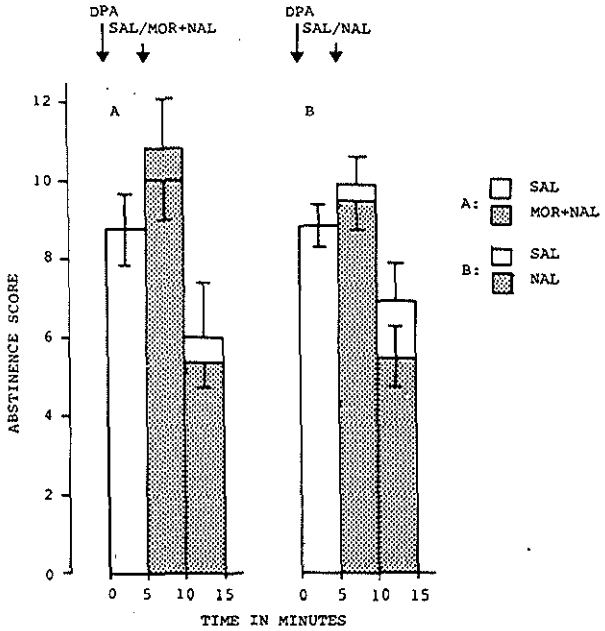


Fig. 3.3.5. Effect of naloxone alone or in combination with morphine on DPA induced abstinence behaviour.
A. Two groups of 6 rats received 300 mg/kg DPA and were treated with saline or 1 mg/kg morphine + 1 mg/kg naloxone. A third group, receiving DPA and 1 mg/kg morphine 5 min later showed the same suppression of abstinence behaviour as shown in Fig. 3.3.4.
B. Two groups of 12 rats were treated with saline or with 1 mg/kg naloxone 5 min later after having received 300 mg/kg DPA. The bars in A end B refer to the mean score per animal ± S.E.

that the increase of GABA concentration in the brain was caused by an inhibition of GABA-transaminase activity (GODIN et al., 1969; CIESIELSKI et al., 1975) studies of HARVEY et al. (1975) indicate that only SSA-dehydrogenase is inhibited. This finding has been recently confirmed by ANLEZARK et al. (1976).
In studies concerning the role of acetylcholine in precipitated abstinence be-

haviour FREDERICKSON (1975) distinguishes between central abstinence signs (shaking behaviour, escape digging, ptosis, penile erection and irritation) and peripheral signs (rhinorrhea, lacrimation, salivation, diarrhoea and seminal emission). The symptoms observed in this study during DPA-induced abstinence behaviour are only characteristic for the first group of abstinence signs. DPA administration increased animal activity in a dose dependent manner which run in parallel with the severity of the syndrome. However, at 50 mg/kg DPA a significant reduction of motor activity was observed. Similar biphasic effects have been described by others (MISSLIN et al., 1975). The fast onset of action of DPA observed is in agreement with experiments in which DPA was used to protect susceptible mice against audiogenic seizures, where maximal protection was obtained between 2.5 and 5 min after administration (SIMLER et al., 1973). Since bicuculline suppressed the observed DPA-induced abstinence behaviour an increased GABA-ergic activity might be held responsible for this behaviour. Administration of bicuculline before DPA did not result in suppression. Possibly, because the duration of the effect of bicuculline is short and restricted by compensatory mechanisms. A similar short acting effect of 2 mg/kg bicuculline in rats is reported by KENT & FEDINETS (1976) who studied the degree of hypothalamic selfstimulation. In our opinion, the suppression of the DPA induced abstinence behaviour by bicuculline together with the known interference of DPA with GABA metabolism, provide evidence to suggest that the behavioural syndrome induced by DPA is the result of an increased availability of GABA at its receptor sites.

The experiments with morphine and naloxone (Fig. 3.3.4 and 3.3.5) demonstrate that morphine receptors are involved in the DPA induced abstinence behaviour. The relatively low dose of 1 mg/kg morphine was sufficient to obtain a clearcut suppression of this behaviour. No signs of sedation were observed under these conditions, thus excluding the possibility that the suppression was caused by some non-specific sedative effect of morphine. Signs of sedation were indeed observed at higher doses of morphine (Table 3.3.1). Under these conditions the DPA-induced increase in locomotor activity was nearly totally inhibited giving only 20% of baseline activity found in saline pretreated rats. Furthermore, the experiments showing that naloxone could release abstinence behaviour in rats previously treated with DPA and morphine (Fig. 3.3.4), at a time where no effect was observed in all control groups, suggests that a close relationship must exist between the effect of DPA and the subsequent suppression by morphine. The mechanism by which morphine counteracted the DPA induced abstinence behaviour is not yet known, but might be explained by an inhibition of GABA release as suggested by COSTA et al. (1975).

Recently it was demonstrated that shaking behaviour in morphine dependent rats after naloxone precipitation was prevented in vitamin B_6 deficient rats (SIU et al., 1976). Since it is known that glutamate decarboxylase activity is

decreased during vitamin B_6 deficiency, leaving GABA-transaminase relatively unimpaired (BAYOUMI et al., 1972), it is conceivable that inhibition of GABA synthesis in morphine dependent rats will inhibit the precipitation of abstinence behaviour or will affect the development of physical dependence. This might support the idea that morphine abstinence behaviour is related to an overactive GABA system. Indeed HO et al. (1976) reported that inhibition of GABA-transaminase by amino-oxyacetic acid accelerated the development of physical dependence, while 2 mg/kg bicuculline inhibited this mechanism. Assuming that the DPA-induced behavioural syndrome is directly related to morphine abstinence, these studies support our conclusion that an interrelationship exists between the GABA system and the development of morphine abstinence behaviour.

A quasi morphine abstinence syndrome has been defined by COLLIER (1974) as "an effect resembling one elicited by withdrawal of a drug on which an animal has been made dependent but provoked by another treatment in a naive animal never exposed to drug nor to like-acting congener that induces such dependence". It is not yet clear whether the DPA-induced abstinence behaviour can be considered as quasi-morphine abstinence behaviour according to these conditions. As already discussed, central abstinence signs were observed representing only one group of abstinence signs according to the classification made by FREDERICKSON (1975). Therefore it can be concluded that an increased GABA-ergic activity may have an important role in that part of the morphine abstinence syndrome.

3.4. EFFECT OF VARYING DOSES OF MORPHINE ON DPA-INDUCED ABSTINENCE

The experiments described in chapters 3.3 and 3.9 indicated that the depressant effects of morphine on activity parameters (see also chapter 1.5) were potentiated considerably in the presence of DPA. Administration of 1 mg/kg morphine to DPA-pretreated rats decreased horizontal activity to levels generally observed in control groups, whereas 2 mg/kg morphine produced a much stronger depression of motor activity. This might indicate that somehow an increased GABA-ergic activity potentiated the depressant effect of morphine on motor activity parameters. Because these depressant effects of morphine might interfere, or may even be responsible for the observed suppression of DPA-induced abstinence behaviour, the following experiments were performed in order to obtain a separation between the depressant effects of morphine alone on motor activity parameters and the suppression by morphine of abstinence behaviour in DPA-pretreated rats.

3.4.1. Experimental

Male Wistar random rats (125 to 200 g of weight were habituated to a new environment for 30 min and injected with 300 mg/kg DPA or saline in a vol of 3 ml/kg via the intraperitoneal route and the behaviour was observed for 5 min. Thereafter, both groups were injected with varying doses of morphine or saline in a vol of 1 ml/kg and subsequently observed for another two 5 min periods. In a second series of experiments morphine and DPA were administered at the same time and the behaviour was observed for three 5 min periods.

3.4.2. Effect of morphine on DPA-induced abstinence behaviour

Morphine suppressed abstinence behaviour in a dose dependent manner during the 10 min after treatment with morphine (Fig. 3.4.1). Abstinence scores were also reduced by morphine in the 5-10 min sub-period (except for 0.5 mg/kg), while in the 10-15 min sub-period only administration of a dose of 2 mg/kg morphine was effective. The effects on saline pretreated rats were not notable, scores being within the range of 1.5 to 2.5. Similar effects of morphine were observed on shaking. The number of shakes decreased from about 10 in the 5-10 min sub-period for the saline treated group to 1 shake after treatment with 2 mg/kg morphine. ED_{50} values for the effects of morphine on abstinence behaviour and shaking were measured using log-dose effect curves as indicated in Fig. 3.4.3. The results gave similar ED_{50} values of 0.55 mg/kg morphine for inhibition of

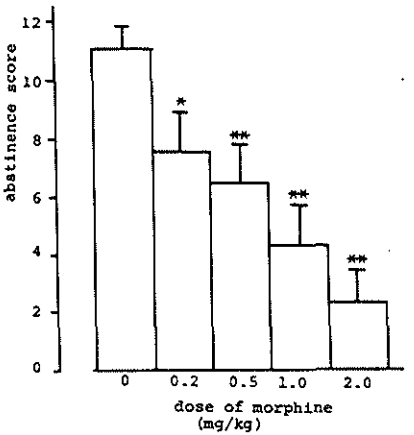


Fig. 3.4.1. The effect of morphine on DPA induced abstinence behaviour. Ten groups of 8 rats were pretreated with saline or 300 mg/kg DPA and received saline or 0.2, 0.5, 1.0 or 2.0 mg/kg morphine after 5 min. Abstinence scores are based upon scores given for the 10 min period after injection of morphine and are the mean ± S.E. for 8 rats. The effect on saline pretreated rats is not indicated in the figure, but these values are all in the range of 1.5 to 2.5. Statistics (Mann Whitney U-test):
*: $p < 0.01$; **: $p < 0.001$, compared with saline treatment.

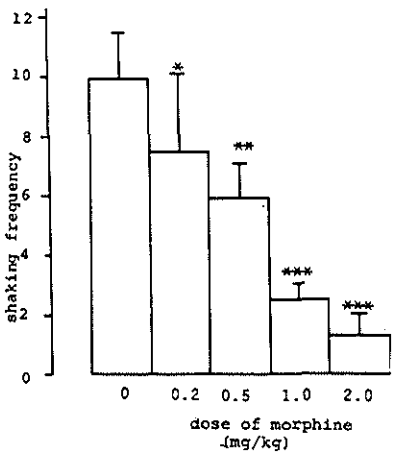


Fig. 3.4.2. The effect of morphine on DPA induced shaking. The experimental details are given in Fig. 3.4.1. The vertical bars refer to the mean score per animal ± S.E. Saline treated rats only occasionally showed this behaviour. Statistics (Mann Whitney U-test, one tailed):
*: $p < 0.052$ (N.B. a highly significant effect was observed in the 5-10 min subperiod for 0.2 mg/kg); **: $p < 0.05$; ***: $p < 0.001$, all compared with saline treated controls.

the responses (abstinence score or shaking frequency) by morphine.

3.4.3. The effect of morphine on motor activity parameters

Activity counts, obtained in the 10 min after injection of morphine in DPA pretreated rats, were depressed by morphine administration in a dose dependent manner both for horizontal and vertical activity (for horizontal activity, see Fig. 3.4.4). Morphine in a dose of 1 mg/kg reduced horizontal and vertical activity, whereas the depression with other doses of morphine was not statistically significant. Calculations of ED_{50} values for the effect of morphine on

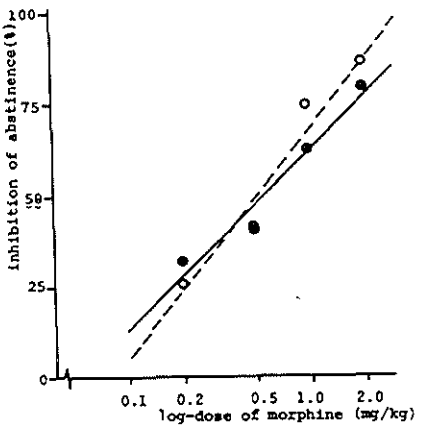


Fig. 3.4.3. Log-dose response curve for the effect of morphine on DPA induced abstinence and shaking behaviour. The percentage inhibition of abstinence (–○–○–) or shaking frequency (–●–●–) was plotted versus log(dose) of morphine.

activity parameters gave approximately the same value as was observed for the effect on abstinence behaviour and shaking frequency, viz. 0.40 and 0.35 for horizontal and vertical activity respectively (Fig. 3.4.5).
The results in Fig. 3.4.4 demonstrate that morphine suppressed abstinence behaviour and horizontal activity in DPA pretreated rats with low doses of 0.2 and 0.5 mg/kg, which had no depressant effect on horizontal activity in saline pretreated rats.
The results in Fig. 3.4.6 show that morphine had pronounced effects on activity parameters during the 10-15 min observation period. Vertical activity was reduced with all doses of morphine, while horizontal activity was only significantly reduced in saline pretreated rats with doses of 1 or 2 mg/kg. A comparison

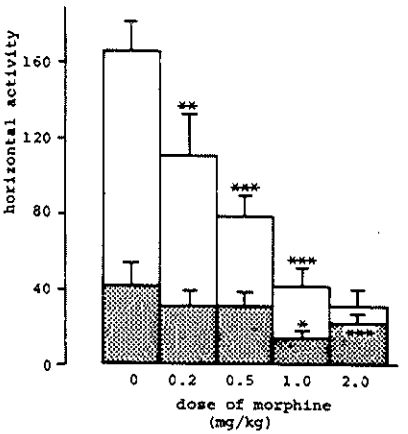


Fig. 3.4.4. Effect of morphine on DPA induced horizontal activity. Horizontal activity was measured during 10 min after injection of morphine or saline. Experimental values: DPA pretreated (open bars) and saline pretreated (shaded bars). Statistics (Mann Whitney U-test, one tailed):
*: $p < 0.025$, compared with the saline treated group for the saline pretreated group; **: $p < 0.01$; ***: $p < 0.001$, both compared with the saline treated group for the DPA treated groups.

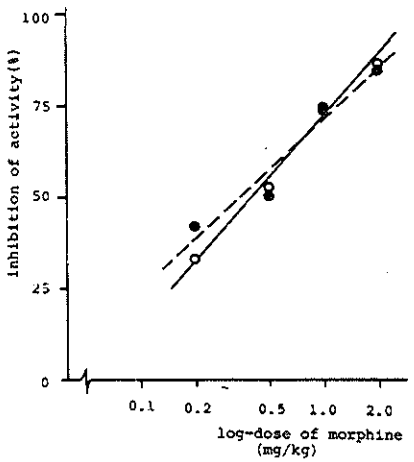


Fig. 3.4.5. Log-dose response curve for the effects of morphine on horizontal and vertical activity in DPA treated rats. The percentage inhibition by morphine of DPA induced horizontal (–○–○–) or vertical (–●–●–) activity was plotted versus the log(dose) of morphine.

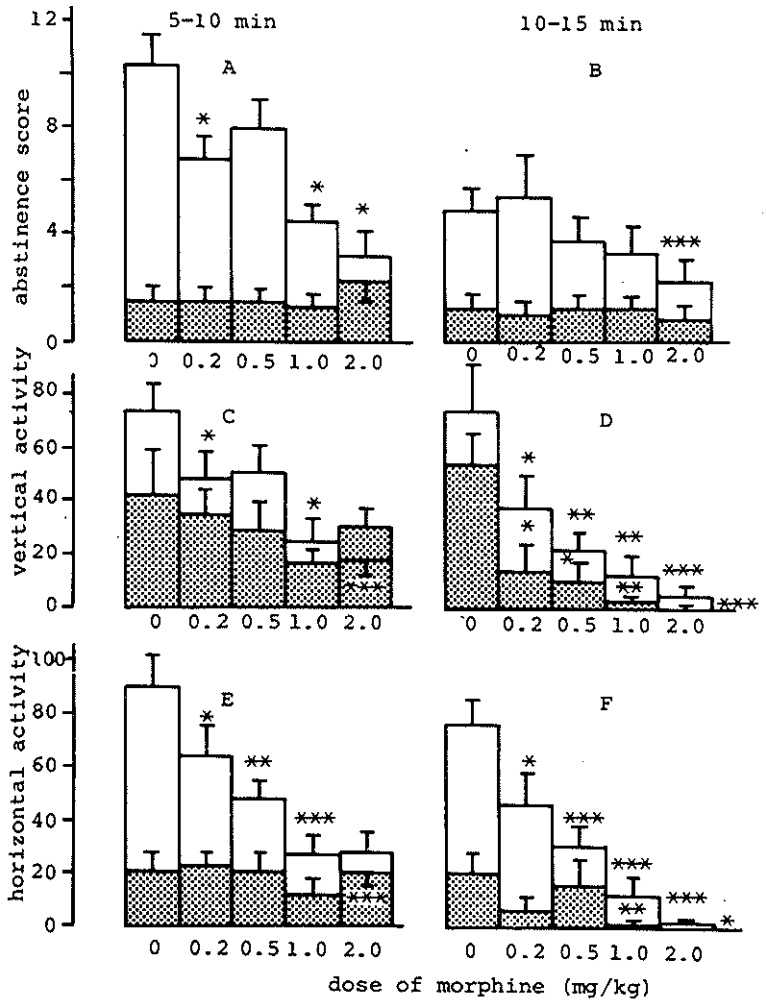


Fig. 3.4.6. Selective effects of morphine on DPA induced abstinence and activity parameters in subperiods of 5 min. The effect of morphine with varying doses on DPA induced abstinence (A and B), vertical activity (C+D) and horizontal activity (E+F) during subperiods 5-10 min (A, C and E) or 10-15 min (B, D and F) are shown. The vertical bars are the mean ± S.E. for groups of 8 rats after pretreatment with saline (shaded area) or DPA (open area). Statistics (Mann Whitney U-test, one tailed).
*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$, compared to appropriate controls treated with saline.

of the effects of morphine on saline as compared to DPA pretreated rats during the two 5 min sub-periods after administration of morphine or saline revealed a differential effect of morphine on saline or DPA treated groups (Fig. 3.4.6). Morphine decreased DPA-induced abstinence behaviour, horizontal and vertical activity in a dose dependent manner in the first 5 min sub-period after the treatment with morphine without significantly effecting abstinence behaviour, horizontal or vertical activity during this period in the saline pretreated groups (Fig. 3.4.6A, C and E). The depressant effect of morphine in the dosage range of 0.2 to 2.0 mg/kg was confined to the 10-15 min observation period when only 2 mg/kg morphine suppressed abstinence behaviour.

3.4.5. Treatment with morphine together with DPA

Comparable results were obtained by morphine administration at the same time as DPA when compared with the treatment with morphine 5 min after DPA. However, the depressant effect of morphine on abstinence behaviour and motor activity was observed with higher doses of morphine as shown by the ED_{50} values summarized in Table 3.4.1.

Table 3.4.1.

The effect of morphine on DPA induced abstinence and motor activity administered together or 5 min after DPA

	together		5 min afterwards	
	ED_{50}	r *	ED_{50}	r *
Abstinence	0.57	0.98	1.15	0.97
Horizontal act.	0.40	0.97	1.15	0.99
Vertical act.	0.35	0.99	0.89	0.92

Morphine was administered together with DPA or 5 min afterwards. The experiments with morphine administration 5 min after administration of DPA have been discussed in the foregoing sections. For the experiments with concurrent administration of DPA and morphine doses of 0.02 to 5 mg/kg were used. ED_{50} values were calculated on the basis of log(dose) response curves using 0.5, 1.0, 2.0 and 5.0 mg/kg morphine.
*: correlation coefficient.

3.4.6. Discussion

Doses of morphine as low as 1 mg/kg have a depressant effect on several behavioural parameters, including locomotor activity (chapter 1.5.1). The present experiments with saline pretreated rats extend these observations indicating that

vertical activity was already suppressed after treatment with 0.2 mg/kg, while horizontal activity was only depressed with 1 mg/kg morphine. This demonstrates the importance of separate measurement of horizontal and vertical activity. Abstinence scores were reduced by morphine with an ED_{50} of 0.5 mg/kg. A comparable ED_{50} was obtained for the effect of morphine on DPA-induced activity, demonstrating the close association of abstinence scores, shaking frequency, horizontal and vertical activity.

Comparison of the effect of morphine on DPA-induced horizontal activity (Fig. 3.4.4), abstinence scores (Fig. 3.4.1) and wet shaking (Fig. 3.4.2) with the effect of morphine on activity parameters in saline pretreated groups (for horizontal activity see Fig. 3.4.4) suggests that the depressive effect of morphine in saline treated rats is not related to the effect on DPA-pretreated rats. The results in Fig. 3.4.4 demonstrate that morphine depressed horizontal activity with 1 mg/kg only, while all doses reduced the scores for abstinence and the number of wet shakes and horizontal activity. This indicates that a general sedative effect of morphine, resulting in a general reduction of all activity parameters does not occur with the low doses of morphine that suppress abstinence behaviour after DPA administration. However, the dissociation is not complete because low doses of morphine suppressed vertical activity in saline pretreated rats (Fig. 3.4.6). A close examination of the depressant effect of morphine in saline pretreated rats indicates that morphine was only effective in the 10-15 min observation period as demonstrated for horizontal and vertical activity in Fig. 3.4.6 C, D, E and F. No significant effect was observed during the 5-10 min observation period. On the other hand, the effect of morphine on DPA-induced abstinence behaviour was observed during the 5-10 min observation period. Therefore, the suppression by morphine of motor activity parameters in saline pretreated rats is not simply related to the inhibition of abstinence behaviour after similar doses of morphine. The observation that the effect of morphine was prevented by concurrent administration of naloxone (chapter 3.3.7), the release of abstinance behaviour by naloxone in DPA and morphine pretreated rats (chapter 3.3.5), together with the observation that the suppressive effect of morphine on motor activity parameters was not causally related to its suppressive effect on DPA-induced abstinence behaviour and motor activity with respect to dose and time, indicates the specificity of the effect of morphine on DPA-induced abstinence behaviour.

It might be argued that morphine administration 30 min before DPA would result in a low ED_{50} for the suppressive effect of morphine on DPA-induced abstinence behaviour, as the time between morphine administration and the complete development of analgesia is about 30 to 40 min. Pilot experiments clearly indicated that this was not the case as some depression of DPA-induced abstinence behaviour was only observed with analgesic doses of 3 to 10 mg/kg morphine. Therefore, the observed effect of morphine on DPA-induced ab-

stinence behaviour can be clearly dissociated from morphine analgesia for two reasons: the time-effect relationship is completely different and the doses required for an analgesic response in control animals are about 10 times higher than the doses of morphine required for suppression of DPA-induced abstinence behaviour. It must be stressed in this respect that the possibility that the analgesic effect of morphine is potentiated by DPA, has not been examined. Some independent experiments indicate that inhibition of GABA-metabolism may potentiate the analgesic effect of morphine (YONEDA et al., 1976) or methadone (KÄARIÄNEN & VIKING, 1976), although others reported hyperalgesia with GABA itself or AOAA administration (WAY, 1973). However, abstinence behaviour is generally associated with a decrease of the pain-threshold. The results with concurrent administration of morphine and DPA, together with some pilot studies mentioned above, suggest that the high sensitivity for morphine observed in this study is somehow related to the time of administration. This may indicate that during DPA-induced abstinence behaviour administration of morphine is more effective than after pretreatment with morphine. However, changes in availability of morphine may occur due to a change in the blood brain barrier for morphine in the presence of DPA, while also the concurrent administration of DPA and morphine may effect the absorption of morphine. Such factors have to be excluded before it can be concluded that the high sensitivity of rats during DPA-induced abstinence behaviour might reflect an increased affinity of opiate receptors for morphine.
In conclusion, the experiments with varying concentrations of morphine have resulted in a clearcut separation of the depressant effects of morphine and the suppressive effect of morphine on DPA-induced abstinence behaviour. This, together with the low ED_{50} observed in the present experiments demonstrates the close association between the effect of morphine and the GABA-ergic system. Some further aspects of the interaction between DPA and morphine will be discussed in chapter 3.9.

3.5. EFFECT OF PICROTOXINE AND STRYCHNINE ON DPA-INDUCED ABSTINENCE BEHAVIOUR

In previous chapters it has been demonstrated that DPA-induced abstinence behaviour is mediated via an increase of GABA at receptor sites. Of the known GABA antagonists two have found widespread acceptance in the pharmacology and electrophysiology of the GABA-ergic system, viz. bicuculline and picrotoxine (KELLY & BEART, 1975). Both GABA antagonists might have their action via different ionic mechanisms (TAKEUCHI & TAKEUCHI, 1969), while only bicuculline is capable of displacing GABA from its binding sites (FISZER DE PLAZAS & DE ROBERTIS, 1975; PECK et al., 1976; YOUNG et al., 1976). A comparison of their action in the present model of increased GABA-ergic activity seemed worthwhile for two reasons: Firstly, it might provide further evidence for an involvement of the GABA-ergic system in DPA-induced abstinence behaviour, and secondly, because bicuculline and picrotoxine may have a different mode of action as mentioned above.
Both bicuculline and picrotoxine are potent convulsant drugs. To exclude the possibility that their convulsions-inducing property was responsible for the effect on the GABA-ergic system, experiments were done with the glycine-antagonist strychnine (RYALL, 1975), which is also a potent convulsant. Strychnine is often used in comparison with bicuculline and picrotoxine to demonstrate the selectivity of the effect of GABA antagonists (MAO et al., 1975; RYALL, 1975; KELLY & BEART, 1975; see also chapter 1.3).

3.5.1. Experimental

Male Wistar random rats (100 to 150 g of weight) were used for these experiments. After a habituarion period of 40 min in a new environment rats were injected with saline or picrotoxine (5 mg/kg, intraperitoneally in a volume of 1 ml/kg) and observed for 10 min. Thereafter, 300 mg/kg DPA or saline was injected and the rats were observed for another 30 min. The following groups were therefore considered: SALxSAL; SALxDPA; PICxSAL and PICxDPA, all consisting of 8 animals.

3.5. PRELIMINARY EXPERIMENTS WITH VARYING DOSES OF PICROTOXINE

Using varying doses between 2 and 5 mg/kg picrotoxine administered between 5 or 20 min before DPA it was observed that only the highest dose was able to suppress abstinence behaviour during the first 10 min after DPA administration.

These preliminary results also indicated that picrotoxine may have some agonist properties as it increased abstinence scores shortly after administration. Therefore it was decided to use 5 mg/kg picrotoxine with a pretreatment time of 10 min to avoid this agonistic action of picrotoxine itself, which might obscure its suspected antagonist properties.

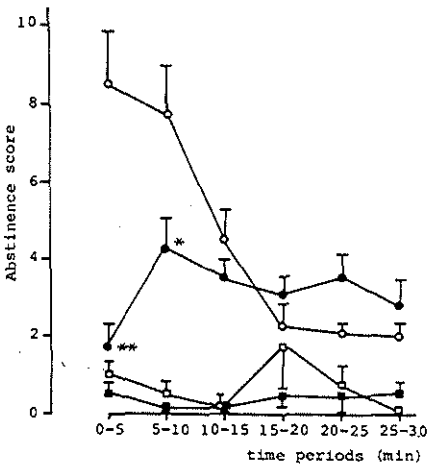


Fig. 3.5.1. Effect of picrotoxine on DPA induced abstinence behaviour. Four groups of 8 rats were injected with saline or 5 mg/kg picrotoxine, observed for 10 min and injected with saline or 300 mg/kg DPA. Therefore, the following groups were considered: SAL x SAL (–□–□–); PIC x SAL (–■–■–); SAL x DPA (–○–○–) and PIC x DPA (–●–●–). All experimental refer to the mean score per animal ± S.E. Statistics (Mann Whitney U-test, two tailed):
*: $p < 0.004$; **: $p < 0.002$, when compared with SAL x DPA.

3.5.3. Effect of picrotoxine on DPA-induced abstinence behaviour

Picrotoxine suppressed the abstinence scores during the first 5 min after administration of DPA to levels of saline or picrotoxine pretreated controls (SALxSAL or PICxSAL). Suppression of DPA-induced abstinence behaviour was also observed during the second 5 min period after DPA administration (Fig. 3.5.1). Abstinence scores after the PICxDPA treatment remained on a fairly constant level between 5 and 30 min after DPA administration, whereas in the SALxDPA-treated group abstinence scores sharply decreased 10 min after DPA injection.

3.5.4. Effect of picrotoxine on DPA-induced motor activity

The effect of picrotoxine on horizontal activity after DPA administration was comparable with that on abstinence behaviour. Horizontal activity was significantly decreased by the picrotoxine pretreatment during the first 5 min after DPA administration, whereas after 25 min horizontal activity was increased (Fig. 3.5.2). The effect of picrotoxine on vertical activity was more

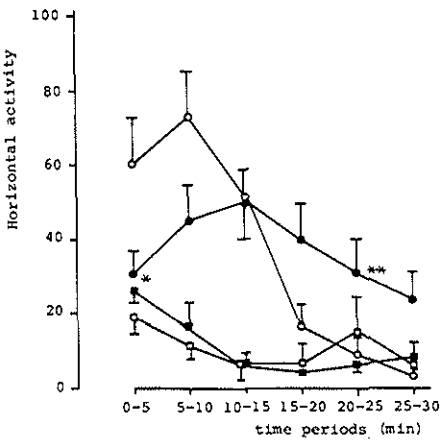


Fig. 3.5.2. Effect of picrotoxine on DPA induced horizontal activity. Experimental details are similar to those described in the legend of Fig. 3.5.1. All points refer to the mean score ± S.E. Statistics (Mann Whitney U-test):
*: $p < 0.05$; **: $p < 0.01$ when compared with SAL x DPA.

pronounced and a complete suppression was obtained during the total observation period. This depression of vertical activity was probably associated with the effect of picrotoxine on vertical activity in saline treated rats (Fig. 3.5.3). Measurement of horizontal or total activity for 30 min after DPA administration indicated that these motor activity parameters were not significantly reduced by the pretreatment with picrotoxine. A significant depression of vertical activity by the pretreatment with picrotoxine was observed in saline as well as DPA-treated groups. These results demonstrate that pretreatment with picrotoxine resulted in a slower onset but longer duration of the increase of horizontal or vertical activity after DPA treatment. As a result no effect of picrotoxine was observed when motor activity parameters were compared for the whole observation period after DPA treatment (Table 3.5.1). Only vertical activity was decreased in this 30 min observation period, probably because picrotoxine also

decreased vertical activity in saline treated rats. The increase of total activity as the result of treatment with DPA (counts in the presence of DPA minus counts in its absence, viz. with saline) was 245.2 counts with saline and 199.4 counts with pretreatment with picrotoxine (see Table 3.5.1), the difference being caused by the smaller increase in vertical activity observed after pretreatment with picrotoxine as compared to saline treatment.

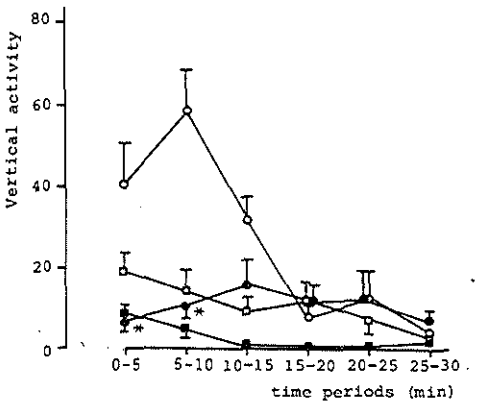


Fig. 3.5.3. Effect of picrotoxine on DPA induced vertical activity. Experimental details are similar to those described in the legend of Fig. 3.5.1. All points refer to the mean score per animal ± S.E. Statistics (Mann Whitney U-test, two tailed): *: $p < 0.002$, when compared with SAL x DPA.

Table 3.5.1.

Effect of picrotoxine on motor activity parameters between 0 and 30 min after DPA administration

Treatment	horizontal activity	vertical activity	total activity
SAL x SAL	66.9 ± 18.7	63.4 ± 10.0	130.1 ± 26.9
PIC x SAL	68.9 ± 9.6	15.5 ± 2.5**	84.4 ± 11.3
SAL x DPA	225.6 ± 38.3	149.8 ± 30.9	375.3 ± 67.5
PIC x DPA	216.0 ± 41.2	63.3 ± 22.7*	283.8 ± 60.6

Experimental details are exactly as described in the legend of Fig. 3.5.1. Motor activity parameters are based on activity counts between 0 and 30 min after administration of DPA. Statistics (Mann Whitney U-test, two tailed):
*: $p < 0.01$, when compared with SAL x DPA; **: $p < 0.004$ when compared with SAL x SAL.

Table 3.5.2.

Effect of picrotoxine on abstinence scores and motor activity parameters

Treatment	time (min)	horizontal activity	vertical activity	total activity	abstinence score	H/V ratio
saline	0 - 5	52.8 ± 5.1	73.1 ± 6.9	125.9 ± 10.2	2.9 ± 0.7	0.79 ± 0.08
picrotoxine	0 - 5	62.5 ± 7.2	65.8 ± 6.8	128.3 ± 13.3	5.3 ± 0.9*	0.96 ± 0.07
saline	5 - 10	22.7 ± 4.6	35.8 ± 7.1	50.1 ± 9.1	1.0 ± 0.3	0.60 ± 0.11
picrotoxine	5 - 10	22.6 ± 3.3	5.0 ± 0.8**	27.6 ± 3.5**	1.5 ± 0.4	4.74 ± 0.46**

These experiments refer to two groups of 16 rats which were treated with picrotoxine or saline and were observed for two 5 min periods. Statistics (Mann Whitney U-test, two tailed):
*: $p < 0.05$; **: $p < 0.004$, when compared with the saline treatment.

3.5.5. Abstinence behaviour and motor activity changes induced by picrotoxine itself

Initial experiments already indicated that injection of picrotoxine itself resulted in considerable digging and shaking behaviour lasting for about 5 min. This observation was confirmed in the complete study, where a significant abstinence score for picrotoxine when compared with saline was obtained. The results are summarized in Table 3.5.2 and indicate that horizontal activity was not affected by picrotoxine. Vertical activity remained initially unaffected, but was decreased to virtually zero in the second 5 min observation period (Table 3.5.2) and also during subsequent observation periods (Fig. 3.5.3). As a result of this effect of picrotoxine on vertical activity, the H/V-ratio increased and total activity decreased during the 5-10 min observation period after treatment with picrotoxine.

3.5.6. Effect of strychnine in DPA-induced abstinence behaviour and motor activity.

Two experiments were performed with strychnine. In one experiment two groups of 5 rats were injected subcutaneously with 0.9 mg/kg strychnine-nitrate, a threshold dose inducing slight myoclonic head twitches in about half of the animals. After 10 min both groups were treated with 300 mg/kg DPA and abstinence behaviour was observed for 15 min.
In a second experiment two groups of 5 rats were injected with 300 mg/kg DPA. After 5 min one group was treated with saline, while a second group received 0.9 mg/kg strychnine-nitrate. The rats were observed for 10 further min.
The results with a threshold dose of strychnine of 0.9 mg/kg (subcutaneously) are summarized in Table 3.5.3, demonstrating that both treatments did not antagonize DPA-induced abstinence behaviour, shaking behaviour, escape digging or motor activity parameters. Instead, in the first experiment strychnine increased the frequency of escape digging although such an effect was not observed with the second experiment. Myoclonic head twitches were observed about 6 min after administration of strychnine and apparently had no effect on abstinence behaviour after DPA administration.

3.5.7. Discussion

Bicuculline and picrotoxine have been proposed as selective antagonists of the interaction of GABA with its receptor (KELLY & BEART, 1975; see also chapter 1.2.14). The experiments performed with bicuculline in chapter 3.3.4

Table 3.5.3.

Effect of strychnine on DPA induced abstinence behaviour and motor activity changes

	treatment before DPA		treatment after DPA	
	SAL x DPA	STR x DPA	SAL x DPA	STR x DPA
a) abstinence behaviour				
abstinence score	10.0 ± 1.7	12.6 ± 3.1	16.6 ± 1.1	14.2 ± 1.4
shaking behaviour	18.2 ± 5.1	19.8 ± 2.3	14.8 ± 2.0	14.0 ± 2.8
escape digging	0.4 ± 2.2†	6.6 ± 2.2†*	15.2 ± 2.1†	10.6 ± 2.7†
b) motor activity				
horizontal activity	180.6 ± 49.9	153.4 ± 34.3	112.6 ± 21.2	96.2 ± 20.1
vertical activity	228.6 ± 55.8	203.0 ± 29.2	115.2 ± 13.9	102.8 ± 17.3
total activity	409.2 ± 102.4	356.4 ± 59.7	227.8 ± 34.0	199.0 ± 36.2

Two groups of 5 rats were pretreated with strychnine or saline and received after 10 min 300 mg/kg DPA (referred to in the table as treatment before DPA). Two other groups of 5 rats were pretreated with 300 mg/kg DPA and received saline or strychnine after 5 min. The first group was observed for 15 min and the figures summarized above are based upon an observation period of 15 min. The second group was observed for the 10 min after treatment with saline of strychnine and the figures are based upon this observation period.

†: the frequency of escape digging is markedly different between the two experimets, which is also reflected in the lower score for abstinence observed in the first experiment. This is caused by the use of saw-dust in the first and sawings in the second experiment. Rats prefer, apparently, sawings to put their head in.

*: $p < 0.05$ (Mann Whitney U-test, one tailed), when compared with saline pretreatment.

indicated that the GABA receptor was involved in the effect of DPA. Since there is considerable debate among electrophysiologists about which is the preferred antagonist to identify a GABA-ergic mechanism, the effect of picrotoxine on DPA-induced abstinence behaviour was studied in addition to that of bicuculline. From initial experiments it became apparent that the time-schedule was very important. The initial GABA-agonistic action of picrotoxine, the time course of the effect of picrotoxine on lateral hypothalamic selfstimulation (KENT & FEDINET, 1976) and the time course of the effect of picrotoxine on c-GMP formation in cerebellum (MAO et al., 1975) indicated that optimal results could be achieved using a pretreatment period of 10 min. MAO et al. (1975) have used changes in cerebellar c-GMP levels as an index for GABA-ergic activity in cerebellum. They have demonstrated that an increase of GABA resulted in a decrease of cerebellar c-GMP content using the intraventricular injection of GABA or the GABA-transaminase inhibitor hydroxylamine. Similarly, the treatment with isoniazid or thiosemicarbazide (both inhibitors of glutamate decarboxylase) resulted in an increase of GABA, probably as the result of an inhibition of GABA-synthesis (COSTA et al., 1976). Moreover, KUPFERBERG et al. (1975) have

demonstrated that the anti-convulsant action of DPA in mice correlated with an increase of cerebellar or cortex GABA and a decrease of c-GMP levels.
Therefore, using the effect of picrotoxine and DPA on cerebellar c-GMP levels as a guideline, a pretreatment time of 10 min in accordance with these effects as well as the effect of picrotoxine on hypothalamic selfstimulation (KENT & FEDINET, 1976) was selected. The present results confirm the involvement of GABA in DPA-induced abstinence behaviour as demonstrated in chapter 3.3. A clearcut suppressive effect was observed under conditions where also maximal effects were observed on c-GMP level in cerebellum and hypothalamic self-stimulation (MAO et al., 1975; KENT & FEDINET, 1976). The relatively high dose of picrotoxine which had to be used in the present experiments to suppress DPA-induced abstinence behaviour and motor activity parameters caused myoclonic head-twitches in 63% of the rats. However, these myoclonic head-twitches were moderate lasting about 10 sec in saline treated and 20 to 30 sec in DPA-treated rats. A similar potentiating effect has been observed by DPA on bicuculline treated convulsions (see chapter 3.9). Similarly occurring head-twitches in strychnine-pretreated rats did not interfere with DPA-induced abstinence behaviour, suggesting that the suppressive effect of picrotoxine was not caused by some unspecific effect associated with the use of convulsant drugs. DPA-induced abstinence behaviour can be suppressed by high doses of antagonists. The effects of picrotoxine on DPA-induced changes in motor activity parameters were similar to the effect on abstinence scores, since a depression of horizontal and vertical activity paralleled the effect on abstinence behaviour. However, with horizontal activity a depression was observed during the first 5 min observation period after the treatment with DPA, while a potentiating effect was observed between 20 and 25 min after DPA-administration. This secondary increase relative to the SALxDPA treatment was significant when compared with the treatment with SALxDPA as well as PICxSAL. Comparing horizontal activity between 0 and 30 min after treatment with DPA showed that picrotoxine did not effect total horizontal activity over this 30 min observation period (225.6 ± 38.3 for SALxDPA versus 216.0 ± 41.2 for PICxPDA treatment). Apparently, picrotoxine delayed the onset of the increase of horizontal activity as well as its return to control levels as demonstrated in Fig. 3.5.2. A very significant effect of picrotoxine was observed on vertical activity in the presence or absence of DPA during this 30 min observation period, but similar results were obtained by picrotoxine administration followed by saline treatment. As a consequence, the increase of vertical activity by DPA administration was similar in picrotoxine or saline pretreated rats. As was the case for horizontal activity, this increase of vertical activity was delayed considerably after pretreatment with picrotoxine as compared with saline pretreatment (Fig. 3.5.3) and less pronounced. A similar conclusion can be drawn for the effect of picrotoxine on DPA-induced abstinence behaviour. There was a significant

retardation of the onset resulting in lower scores during the 0-5 and 5-10 min observation period, while between 15 and 30 min after DPA administration, higher scores were obtained in PICxDPA versus SALxDPA treated groups. By comparing the scores with those of their respective control groups, a difference of 1 unit was found for SALxDPA (2.8 ± 0.5) and SALxSAL (1.8 ± 1.0) while a difference of 4.3 units was found for PICxDPA (4.8 ± 0.9) versus PICxSAL (0.5 ± 0.3). This suggests that the pretreatment with picrotoxine prolonged the duration of DPA-induced abstinence behaviour, which is also suggested by the results in Fig. 3.5.1 demonstrating that abstinence scores after PICxDPA reached a constant level between 5 and 30 min after DPA-treatment, whereas the abstinence score for the SALxDPA treated group sharply decreased between 10 and 20 min after administration of DPA.

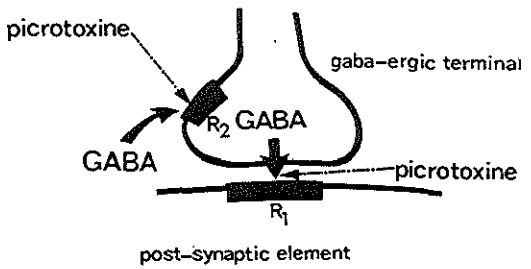


Fig. 3.5.4. Picrotoxine-sensitive presynaptic inhibitory auto-receptors located on GABA-ergic terminals inhibiting the release of GABA.

The unexpected finding that picrotoxine induced abstinence behaviour which could be clearly distinguished from the effects of the saline treatment, is difficult to explain. Similar effects were not noted with bicuculline, although some indication of a potentiating effect of bicuculline on shaking behaviour was observed with a convulsant dose of 3 mg/kg bicuculline. Although this behaviour after picrotoxine cannot be classified as quasi-morphine abstinence behaviour because the effect of a combined treatment with morphine and picrotoxine has not been investigated, these results might indicate that picrotoxine caused an increased release of GABA shortly after administration. COLLINS (1974) observed that bicuculline increased and picrotoxine decreased the electrically evoked release of GABA from isolated spinal cord of the rat. This observation is not in accordance with the agonistic effect of picrotoxine observed in this study. An explanation of this effect might be the presence of GABA-ergic inhibitory receptors on GABA-containing terminals as demonstrated in Fig. 3.5.4. Such a model might be in accordance with an hypothesis of COSTA et al. (1975)

for the action of bicuculline and picrotoxine, where pre-synaptic receptors are selectively sensitive for picrotoxine and bicuculline, but not strychnine.
The experiments with strychnine, a selective glycine antagonist (RYALL, 1975), demonstrate that the antagonism of DPA-induced abstinence is not related to the convulsions-inducing properties of bicuculline and picrotoxine. Moreover, the absence of an effect of the myoclonic head-twitches on DPA-induced abstinence behaviour suggests that these twitches as such do not cause antagonism of DPA-induced abstinence behaviour and therefore probably did not interfere with DPA-induced abstinence behaviour in the present experiments with picrotoxine.
In conclusion, these experiments with picrotoxine and strychnine indicate that a selective blockade of GABA-ergic receptors may reduce abstinence behaviour after DPA administration. Therefore, these results support the concept of a mediation of DPA-induced abstinence behaviour by the GABA-ergic system. The observed initial abstinence-like behaviour observed after administration of picrotoxine might be explained by a concept of picrotoxine sensitive GABA-receptors on presynaptic GABA-ergic terminals which will be discussed in chapter 4.5.

3.6. EFFECT OF GABA-TRANSAMINASE INHIBITION BY AMINO-OXY-ACETIC ACID ON DPA-INDUCED ABSTINENCE BEHAVIOUR

In the previous chapters it was shown that abstinence behaviour could be induced in naive rats by injecting DPA. The evidence that this behaviour was caused by an increased concentration of GABA at receptor sites was based on the findings that it could be suppressed by the selective GABA antagonists bicuculline and picrotoxine, whereas strychnine was not effective. The inhibition of GABA degradation by DPA (HARVEY et al., 1975; ANLEZARK et al., 1976) and the observed increase of GABA *in vivo* can be considered as additional evidence for the involvement of the GABA-ergic system in this kind of behaviour (GODIN et al., 1969; CIESIELSKI et al., 1975).
For quite some time GABA-transaminase inhibition has been considered to be the main biochemical effect of DPA (SIMLER et al., 1973; FOWLER et al., 1975). Recent studies, however, indicate that DPA inhibits SSA-dehydrogenase selectively while having no effect on GABA-transaminase (HARVEY et al., 1975; ANLEZARK et al., 1976). Inhibition of SSA-dehydrogenase by DPA *in vivo* will result in an increased formation of GABA from SSA via the transamination reaction in the presence of an active GABA-transaminase. Therefore, inhibition of SSA-dehydrogenase may cause an increase of GABA concentration in the presence of an unimpaired synthesis of GABA from glutamate by the action of glutamate decarboxylase. Therefore, the effect of inhibition of GABA-transaminase was studied using the GABA-transaminase inhibitor AOAA (VAN GELDER, 1966; WOOD & PEESKER, 1973) to find out whether inhibition of GABA-transaminase can also evoke abstinence behaviour in the rat. Moreover, if GABA accumulation by inhibiting SSA-dehydrogenase requires the presence of an unimpaired GABA-transaminase, it might be expected that AOAA might affect DPA-induced abstinence behaviour. From studies demonstrating a maximal rate of GABA accumulation using 25 mg/kg AOAA (VAN GELDER, 1966; WOOD & PEESKER, 1973) it was concluded that GABA-transaminase would be completely inhibited in rats treated with this dose of AOAA. Because a slight inhibition of glutamate decarboxylase was observed under these conditions (WOOD & PEESKER, 1973) it was preferable to avoid inhibition of glutamate decarboxylase by using lower doses of AOAA, although experiments with thiosemicarbazide indicated that inhibition of GABA-synthesis *in vivo* did not modify DPA-induced abstinence behaviour (see chapter 3.7).

3.6.1. Experimental

Male Wistar random rats were used weighing between 125 and 200 g. After habituation to a new environment for 40 min, the rats were pretreated with saline

or AOAA. After 20 min DPA or saline was injected and abstinence behaviour and activity were measured. In a second experiment rats were treated with 12.5 mg/kg AOAA and observed for 15 min immediately after the injection of AOAA or saline to see whether abstinence behaviour was evoked by AOAA.

3.6.2. Preliminary experiments with AOAA

A complete suppression of DPA-induced abstinence behaviour was observed using 25 mg/kg AOAA. With this dose, however, motor incoordination probably resulting from a paralysis of the hindlimbs as reported by GRIMM et al. (1975) was observed, while the combined treatment with AOAA and DPA 20 min later resulted in a depression of motor activity and hypothermia (about 34°C was noted). Since under these conditions no abstinence behaviour could be measured the dose of AOAA was considerably decreased as described below.

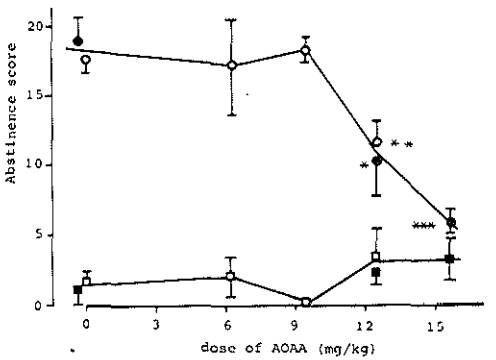


Fig. 3.6.1. Effect of varying doses of AOAA on DPA induced abstinence behaviour. After a habituation period of 40 min in a new environment rats were treated with saline, 6.3, 9.4 or 12.5 mg/kg AOAA and treated after 20 min with 300 mg/kg DPA or saline. All treatments were performed using groups of 6 rats except for SAL x DPA, for which 8 rats were used. A second series of rats was injected with saline, 12.5 or 15.7 mg/kg AOAA and 20 min later with saline or 300 mg/kg DPA, all treatments being performed with groups of 6 rats. Experimental points refer to the mean score per animal ± S.E.: DPA treated, series I (–○–○–) and series II (–●–●–), saline treated, series I (–□–□–) and series II (–■–■–). Statistics (Mann Whitney U-test, two tailed):
*: $p < 0.05$; **: $p < 0.02$; ***: $p < 0.002$, all when compared with saline treatment.

3.6.3. Effect of varying doses of AOAA on DPA-induced abstinence behaviour

The results in Fig. 3.6.1. show that AOAA itself did not produce abstinence be-

haviour but was able to inhibit DPA-induced abstinence behaviour using 12.5 or 15.7 mg/kg AOAA. The time course of abstinence behaviour (Fig. 3.6.2) after DPA administration demonstrates that 12.5 mg/kg AOAA suppressed DPA-induced abstinence behaviour in the 0-5 and 5-10 min observation periods, whereas 15.7 mg/kg AOAA suppressed the DPA-induced abstinence to levels observed after treatment with AOAA alone. No significant difference was observed between DPA or saline indicating that this dose of AOAA completely suppressed DPA-induced abstinence behaviour. The increase in abstinence scores by AOAA administration as compared to saline, might indicate that some abstinence behaviour was observed with AOAA alone.

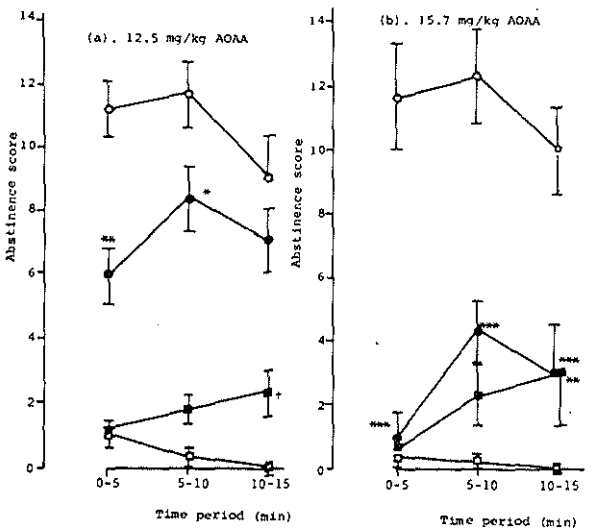


Fig. 3.6.2. The time course of DPA induced abstinence behaviour after pretreatment with 12.5 or 15.7 mg/kg AOAA. The following treatments were studied: SAL x DPA (–○–○–); AOAA x DPA (–●–●–); SAL x SAL (–□–□–) and SAL x DPA (–■–■–). Animals per group: 14 rats for 12.5 mg/kg + DPA; 13 rats for SAL x DPA and 12 rats per treatment for the other two groups in the case of 12.5 mg/kg AOAA experiment; 6 rats for each group in the case of 15.7 mg/kg AOAA experiment. Statistics (Mann Whitney U-test, two-tailed):
*: $p < 0.05$; **: $p < 0.02$; ***: $p < 0;002$, when compared with SAL x DPA treatment; †: $p < 0.02$, when compared with SAL x SAL treatment.

3.6.4. Effect of varying doses of AOAA on DPA-induced motor activity

The effects of pretreatment with AOAA on DPA-induced motor activity were similar to those observed for DPA-induced abstinence behaviour. Treatment

with 12.5 mg/kg or 15.7 mg/kg suppressed DPA-induced horizontal activity as shown in Fig. 3.6.3. Treatment with 6.3 or 9.4 mg/kg AOAA reduced locomotor activity in saline treated rats. However, this depressive effect of AOAA did not interfere with abstinence behaviour nor did it decrease the DPA-induced increase in motor activity parameters. No decrease in horizontal activity was observed with AOAA alone using doses of 12.5 or 15.7 mg/kg, while a suppression of DPA-induced horizontal activity was still observed using these doses. Comparable results were obtained for vertical activity (not shown).

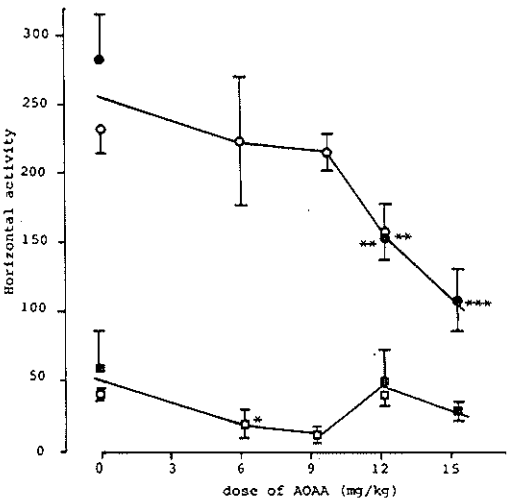


Fig. 3.6.3. The effect of varying doses of AOAA on DPA induced horizontal activity. The results refer to the same experiment as described in Fig. 3.6.1., where further details are described. Experimental points indicate the mean ± S.E.: DPA treated, series I (–○–○–) and series II (–●–●–); saline treated, series I (–□–□–) and series II (–■–■–). Statistics (Mann Whitney U-test, two tailed):
*: $p < 0.05$; **: $p < 0.02$; ***: $p < 0.005$, when compared with saline treatment.

3.6.5. Effect of AOAA on the time course of motor activity after administration of DPA

The effect of AOAA pretreatment on the time course of horizontal activity after administration of DPA was similar to that of AOAA on DPA-induced abstinence behaviour as is shown in Fig. 3.6.4. A significant reduction of horizontal activity was observed by the pretreatment with 12.5 mg/kg AOAA on the first two 5 min observation periods, whereas with 15.7 mg/kg AOAA horizontal activity was decreased in all three observation periods. Both treatments decreased hori-

zontal activity during the first 5 min observation period in saline treated rats, whereas no effect in saline treated rats was observed during the second and third observation period.

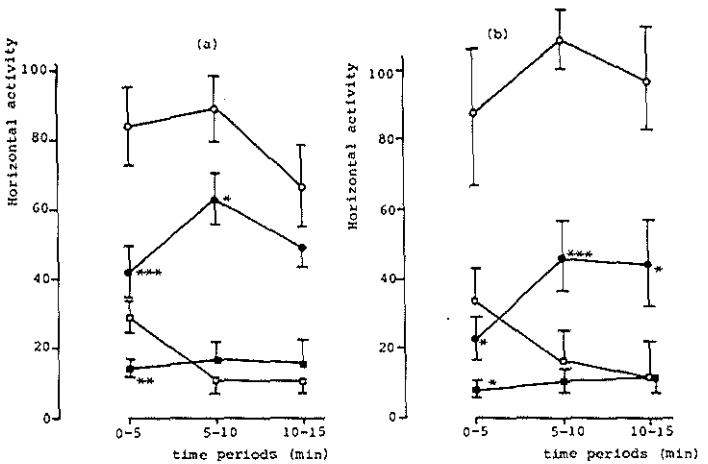


Fig. 3.6.4. The time course of the effects of 12.5 or 15.7 mg/kg AOAA on DPA induced motor activity. The effect of AOAA with 12.5 (left) or 15.7 mg/kg (right) on horizontal activity is shown using similar experimental conditions as described in Fig. 3.6.1. Experimental points refer to the mean ± S.E. for the following treatments: SAL x DPA (–○–○–); AOAA x DPA (–●–●–); SAL x SAL (–□–□–) or AOAA x SAL (–■–■–). Statistics (Mann Whitney U-test, two tailed):
*: $p < 0.05$; **: $p < 0.02$; ***: $p < 0.002$, when compared with SAL x DPA;
†: $p < 0.02$, when compared with SAL x SAL.

3.6.6. Effect of AOAA on the H/V-ratio with and without DPA-treatment

AOAA-treatment increased the H/V-ratio considerably in saline treated rats, while the H/V-ratio remained constant in the DPA treated groups. The results in Table 3.6.1 show that pretreatment with AOAA of the saline treated groups increased the H/V-ratio when compared with saline. DPA alone increased the H/V-ratio from 0.274 to 0.755 while pretreatment with AOAA of the DPA-treated group did not change the H/V-ratio when compared with the DPA-treatment alone, probably because the H/V-ratio was already affected by AOAA alone.

Table 3.6.1.

Effect of AOAA and DPA on the H/V-ratio

pretreatment (mg/kg)	treatment after 20 min (N)	
	saline	300 mg/kg DPA
saline	0.274 ± 0.052 (12)	0.755 ± 0.042*(12)
AOAA (12.5)	0.939 ± 0.187*(12)	0.767 ± 0.069 (12)
AOAA (15.7)	0.475 ± 0.107 (5)	0.998 ± 0.159 (6)

The H/V-ratio was based on the motor activity counts during the 15 min after administration of saline or DPA. Statistics (Mann Whitney U-test, two tailed):
*: $p < 0.002$.

3.6.7. The effect of AOAA on the DPA-induced abstinence signs shaking and escape digging

Apart from the effect of the pretreatment with 12.5 or 15.7 mg/kg AOAA on DPA-induced abstinence scores, significant effects were also observed on DPA-induced shaking and escape digging (Table 3.5.2). The frequency of shaking was significantly affected with 15.7 mg/kg AOAA, while an effect on escape digging was observed using 12.5 as well as 15.7 mg/kg AOAA.

Table 3.6.2.

The effect of AOAA pretreatment on DPA induced digging and shaking

	pretreatment (mg/kg)	(N)	frequency of shaking	frequency of diggin
a.	saline	13	19.2 ± 1.8	15.2 ± 3.6
	AOAA (12.5)	14	14.3 ± 2.8	4.9 ± 3.1*
b.	saline	6	20.3 ± 2.9	18.0 ± 4.6
	AOAA (15.7)	6	5.0 ± 1.5**	2.0 ± 0.7*

The frequency of digging or shaking was measured during 15 min after treatment with DPA with saline or AOAA pretreatment. Statistics (Mann Whitney U-test, two-tailed):
*: $p < 0.004$; **: $p < 0.002$, when compared with saline pretreatment.

3.6.8. Effect of AOAA alone on abstinence behaviour and motor activity

In a separate experiment the effect of 12.5 mg/kg AOAA was studied on abstinence behaviour and motor activity parameters to compare the effect of AOAA

alone with that of DPA. Two groups of 7 rats were injected with AOAA or saline and the behaviour was observed for 15 min as described for DPA. The results in Table 3.6.3 demonstrate that AOAA treatment failed to provoke abstinence behaviour, but decreased the scores when compared with the saline treated group. The incidence of digging was significantly decreased from 5 out of 7 to zero out of 7 rats in the saline and AOAA-treated group, respectively (Fisher's exact probability test, two tailed: $p < 0.05$). Although the H/V-ratio showed a tendency to be decreased by the treatment with AOAA, from 0.547 ± 0.088 to 0.336 ± 0.027, this difference did not reach a level of significance.

Table 3.6.3.

Effect of AOAA alone on abstinence scores when compared with saline

treatment (mg/kg)	(N)	observation period	
		0 - 5 min	0 - 15 min
saline	(7)	4.3 ± 1.3	4.6 ± 1.0
AOAA (12.5)	(7)	1.1 ± 0.6*	2.0 ± 0.8*

Two groups of 7 rats were treated with 12.5 mg/kg AOAA or saline and observed for 15 min. Statistics (Mann Whitney U-test, two-tailed):
*: $p < 0.10$, which is not sufficiently significant when compared with saline treatment.

3.6.9. Discussion

The purpose of the experiments with AOAA was to demonstrate that the effect of AOAA on the behaviour of the rat was different from that observed after treatment with DPA. A second purpose was to see whether an unimpaired GABA-transaminase was required for the expression of the effect of DPA. AOAA was chosen for these experiments since its action is mainly GABA-transaminase inhibition. So far, only two selective inhibitors of GABA-transaminase *in vivo* have been described: ethanolamine-O-sulphate, which does not penetrate the brain and must be given intraventricularly, and γ-vinyl-GABA (FOWLER & JOHN, 1972; FOWLER, 1973; JUNG & METCALF, 1975; JUNG et al., 1976). The latter compound selectively affected GABA-transaminase without modifying glutamate decarboxylase or SSA-dehydrogenase. However, this drug was not available for experiments.

Since AOAA was the only drug available on the moment the effect of this GABA–transaminase inhibitor was investigated. The results demonstrate that abstinence behaviour induced by DPA was suppressed by 12.5 or 15.7 mg/kg AOAA pretreatment, while parallel effects were observed on motor activity

parameters. Treatment with AOAA and saline resulted in a decrease of horizontal activity for all doses during the first 5 min observation period. Apparently, this decrease of horizontal activity did not interfere with DPA-induced abstinence behaviour, since the treatment with 6.3 or 9.4 mg/kg AOAA did not affect abstinence behaviour, although horizontal activity was decreased during the first 5 min observation period. Moreover, using 12.5 or 15.7 mg/kg AOAA, abstinence behaviour was reduced during the first two or all three observation periods, respectively (Fig. 3.6.1), while horizontal activity in saline treated rats was only affected by AOAA during the first 5 min observation period (Fig. 3.6.4). Therefore it seems unlikely that the observed decrease of horizontal activity observed with AOAA during the first 5 min observation period was related to the suppressive effect of AOAA on DPA-induced abstinence behaviour and motor activity changes.

The results in Fig. 3.6.1 demonstrate that the treatment with 12.5 mg/kg AOAA and saline resulted in significant higher scores when compared with saline treated controls. Therefore, a separate study was performed to see whether this dose of AOAA could produce abstinence behaviour comparable with that induced by DPA. The data in Table 3.6.3 show that AOAA itself did not produce abstinence behaviour, but, instead reduced the scores when compared with saline treated rats. Therefore it is concluded that GABA-transaminase inhibition by AOAA treatment does not produce abstinence behaviour and increased motor activity. Inhibition of SSA-dehydrogenase by the treatment with DPA, on the contrary, results in abstinence behaviour and increased motor activity. This suggest that GABA-transaminase and SSA-dehydrogenase may have different regulatory functions in the control of GABA-degradation.

Furthermore, it is concluded that a functionally intact GABA-transaminase is a prerequisite for the occurrance of the DPA-induced abstinence syndrome. These results exclude the possibility that the behavioural effect of DPA *in vivo* is related to inhibition of GABA-transamination. These differential results with AOAA and DPA are not compatible with a simple one-compartment model because AOAA can inhibite GABA-degradation without producing abstinence behaviour whereas DPA can. The existance of two different GABA compartments in the brain must be assumed to explain these differential effects of AOAA and DPA on animal behaviour. The observed inhibition of DPA-induced abstinence behaviour by AOAA suggests that these two GABA-ergic systems have opposite effects, while being closely connected.

The existance of at least two GABA compartments in the brain with quite different properties has been proposed on the basis of compartmentation studies by several authors (VAN DEN BERG et al., 1975; BAXTER, 1976), while it has been suggested that these different compartment might be partially similar to glial and neuronal tissue, respectively (BALÁZS et al., 1973b). In the introductory section, chapter 1.4, evidence has been presented that GABA-trans-

aminase and SSA-dehydrogenase might be located in glial cells and nerve endings, whereas glutamate decarboxylase is almost exclusively located in the neuronal compartment. A model has been presented in which it is suggested that SSA might have a low affinity for SSA-dehydrogenase in the neuronal compartment. This results in a preferential conversion of SSA via the transaminase reaction into GABA. This means that SSA-dehydrogenase would be the rate limiting degradation step in the neuronal compartment, which might contribute to the accumulation of GABA in nerve endings. On the other hand, SSA might have a high affinity for SSA-dehydrogenase in the glial compartment and therefore would result in a non-rate-limiting conversion of SSA preferentially into succinate (see also chapter 2.7).

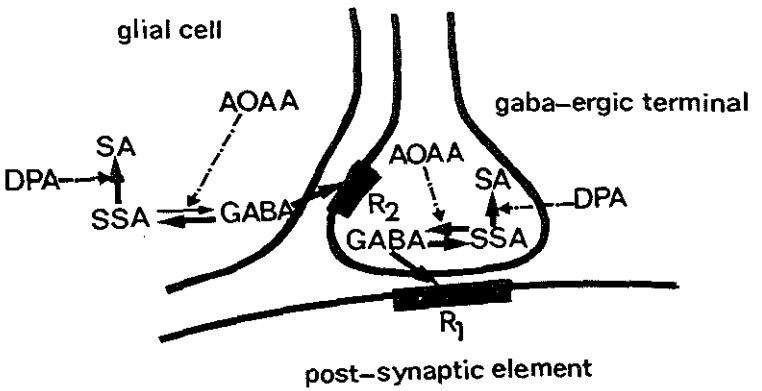


Fig. 3.6.5. GABA-sensitive presynaptic inhibitory auto-receptor affected by release of GABA from glial cells after inhibition of GABA degradation by AOAA. This release of GABA from glial cells inhibits the release of accumulated GABA from nerve terminals after DPA administration, but also after administration of AOAA. This model is similar to that described in chapter 3.5, Fig. 3.5.4.

This model may account for the observation in the present study that AOAA and DPA have differential effects on the behaviour of the rat. According to this model AOAA might inhibit GABA metabolism preferentially in that compartment where GABA-transaminase is probably rate limiting. As a consequence GABA will accumulate rapidly in this compartment after treatment with AOAA and is probably released. This release of GABA from glial elements may presynaptically affect GABA-ergic nerve terminals. On the other hand, DPA may increase GABA in the nerve ending containing neuronal compartment. The suppressive effect of AOAA on DPA-induced abstinence behaviour can be explained by this model. Administration of DPA will increase GABA in nerve ter-

minals both in the presence or absence of AOAA, but its release is prevented by the increased release of GABA from glial cells resulting from the inhibition of GABA degradation by AOAA pretreatment. Apart from inhibiting the glial GABA-transaminase selectively, the model is also valid if only DPA acts preferentially in the neuronal compartment. Although AOAA may inhibit GABA-transaminase in nerve endings thus increasing the neuronal GABA, the presynaptic inhibition by GABA, released from glial cells, will prevent the release of the accumulated GABA in nerve terminals. As a result AOAA will not produce abstinence behaviour as observed in the present study. Moreover, this model also accounts for the inhibitory action of AOAA on DPA-induced abstinence behaviour. In the presence of AOAA accumulation of GABA may occur but its release will be prevented by the inhibitory effect of GABA on the GABA containing terminals, released from glial cells and resulting from the inhibition of GABA degradation by AOAA.

Inhibition of GABA-transaminase *in vivo* using doses of 20 to 40 mg AOAA/kg resulted in an accumulation of GABA with a rate of about 7 μMol/g tissue/h. Similar fluxes have been found using compartmentation studies with labeled glucose (PATEL et al., 1974) and it was demonstrated that both GABA compartments had about similar fluxes. If AOAA only affected the glial compartment, the rate of GABA accumulation would have been half the value estimated from compartmentation studies with labelled precursors. Therefore it is concluded that AOAA probably affected both compartments. This conclusion is supported by the observation by SELLSTRÖM et al. (1975) that AOAA inhibited GABA-metabolism equally effective in both glial and neuronal (nerve endings containing) fractions. The observed selective effect of AOAA on nerve endings derived GABA-transaminase as compared to glial derived GABA-transaminase observed in the same study is not in agreement with the above mentioned experiment and might be only relevant *in vitro* (SELLSTRÖM et al., 1975).

In conclusion, the present results suggest that inhibition of GABA-transaminase or SSA-dehydrogenase have opposite effects *in vivo*, although both increase GABA in the brain (VAN GELDER, 1966; GODIN et al., 1969; WOOD & PEESKER, 1973; CIESIELSKI et al., 1975). Therefore, the present results can only be explained by a compartmented model for GABA in the brain, in which at least DPA may affect one compartment selectivity. These results do not support the suggestion that the pharmacological effects of DPA are caused by inhibition of GABA-transaminase as suggested by Mandel & coworkers (GODIN et al., 1969; SIMLER et al., 1973; CIESIELSKI et al., 1975) and FOWLER (1975). Therefore, these results extend the *in vitro* results of HARVEY et al. (1975) and ANLEZARK et al. (1976) suggesting that the increase of GABA in the brain after treatment with DPA is caused by inhibition of SSA-dehydrogenase.

3.7. EFFECT OF GLUTAMATE DECARBOXYLASE INHIBITION ON DPA-INDUCED ABSTINENCE

In this chapter experiments are described with thiosemicarbazide (TSC) and 3-mercaptopropionate (3-MPA), both inhibitors of glutamate decarboxylase, to study the effect of GABA synthesis inhibition on DPA-induced abstinence behaviour.

TSC administration in a convulsant dose of 25 mg/kg results in a decrease of the concentration of PLP in the brain within 15 min to 40% of control values (BILODEAU, 1965). Under these conditions convulsions occurred with a latency of 65 min. BAXTER (1969) studied the mode of action of TSC by measuring the activity of glutamate decarboxylase, GABA-transaminase, the incorporation of ^{14}C from ^{14}C-glutamate into GABA, and the concentration of GABA. The incorporation of label into GABA was reduced and GABA-levels were decreased, whereas GABA-transaminase was unimpaired, suggesting that TSC selectively inhibits the synthesis of GABA while having no effect on GABA-transaminase. Moreover, a decrease of GABA has been demonstrated in samples taken from the brains of TSC treated dogs (ROA et al., 1964). The involvement of the PLP-cofactor in the action of TSC was demonstrated by the immediate return of glutamate decarboxylase to control levels after injection of the PLP-precursor pyridoxal. Thiosemicarbazone (a complex of TSC and PLP) was ineffective in mice, suggesting that the inhibition by TSC was solely caused by PLP depletion (TAPIA et al., 1969).

The other inhibitor of glutamate decarboxylase used in the present study was 3-MPA. It is a competitive inhibitor of glutamate decarboxylase, thus excluding a role of PLP-depletion in the inhibitory effect of 3-MPA (SPRINCE et al., 1969; LAMAR, 1970; HORTON & MELDRUM, 1973; KARLSSON et al., 1974). It has been suggested that a complete inhibition of glutamate decarboxylase might be obtained using 35 to 180 mg/kg 3-MPA (KARLSSON et al., 1974). Conflicting data exist about the effect of 3-MPA on GABA-transaminase. RODRÍGUEZ DE LORES ARNAIZ et al. (1972) described that glutamate decarboxylase activity was inhibited and GABA-transaminase activity was activated by 3-MPA. The latter effect of 3-MPA was present from 2 to 15 min after administration of 35 mg/kg 3-MPA. Using purified GABA-transaminase SCHOUSBOE et al. (1974) demonstrated that GABA-transaminase was competitively inhibited by 3-MPA *in vitro*. A similar competitive inhibition of GABA-transaminase was reported by LAMAR (1970) using *pseudomonas* GABA-transaminase. The decrease of GABA concentration observed in several brain regions 4 min after administration of 90 mg/kg 3-MPA observed just prior to the occurrance of convulsions suggests a predominant action of 3-MPA on glutamate decarboxylase *in vivo* (KARLSSON et al., 1974).

3.7.1. Experimental

Male Wistar random rats between 100 and 200 g were used for these experiments. After a habituation period of 40 min in a new environment the rats were pretreated with 3-MPA or TSC with saline as a control and injected with 300 mg/kg DPA 1 or 20 min later. From pilot studies it appeared that pretreatment with 3-MPA in doses lower than 25 mg/kg was unable to prevent DPA-induced abstinence behaviour, while increasing the dose produced sever running fits. Therefore, 3-MPA was administered 1 min before DPA to take advantage of the anticonvulsant action of DPA, allowing the use of convulsant doses of 3-MPA.

3.7.2. The effect of thiosemicarbazide on DPA-induced abstinence behaviour

In a first series of experiments rats were pretreated with 25 mg/kg or saline and were injected with 300 mg/kg DPA or saline 20 min thereafter. When compared with saline no effect was observed by the pretreatment with TSC on DPA-in-

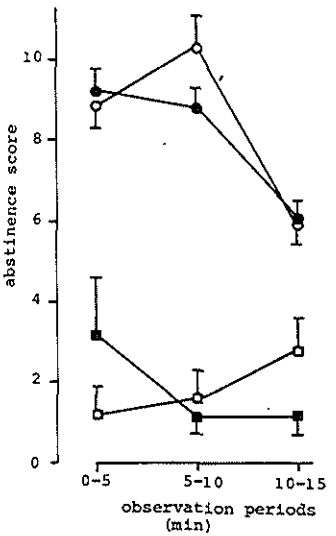


Fig. 3.7.1. Effect of pretreatment with TSC on DPA induced abstinence behaviour. Two groups of 13 rats were pretreated with saline or 25 mg/kg TSC and received 300 mg/kg 1 min later. Abstinence behaviour was observed for three 5 min sub-periods. Similarly, two groups of 5 rats were treated with TSC or saline, while receiving saline 1 min later. The results refer to the mean score per animal ± S.E. Thus, the following groups were considered: SAL x DPA (–○–○–); TSC x DPA (–●–●–); SAL x SAL (–□–□–) and TSC x SAL (–■–■–).

duced abstinence behaviour, shaking or digging frequency, or motor activity parameters. Results obtained during sub-periods of 5 min showed no effect either. All rats convulsed after about one hour and died subsequently, except for one DPA pretreated rat which survived the convulsions.
In a second series of experiments the time between injection of TSC and DPA was reduced to 1 min. The results shown in Fig. 3.7.1. indicate that TSC did not interfere with DPA-induced abstinence behaviour or motor activity changes.

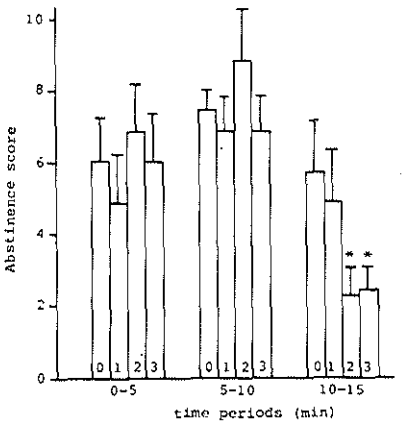


Fig. 3.7.2. The effect of 3-MPA on DPA induced abstinence behaviour. Four groups of 7 rats were pretreated with saline (0), 25 (1), 30 (2) or 35 (3) mg/kg 3-MPA and received 300 mg/kg DPA 1 min later. The bars refer to the mean score per animal ± S.E. Statistics (Mann Whitney U-test, one tailed):
*: $p < 0.01$, when compared with saline.

3.7.3. Effect of 3-mercaptopropionate on DPA-induced abstinence behaviour

Preliminary experiments indicated that doses of 25 mg/kg 3-MPA or more resulted in running fits of variable duration which were followed by a depressed state. It was concluded that these running fits should be avoided if reliable results were to be obtained. However, after treatment with a subconvulsive dose of 20 mg/kg there was no effect on DPA-induced abstinence behaviour. Therefore it was decided to study the effect of convulsant doses in the presence of the anti-convulsant DPA at the time when running fits occurred, i.e. about 6 min after 3-MPA administration. Consequently, 3-MPA was injected 1 min before the challenge with DPA. The results shown in Fig. 3.7.2. indicate that 30 or 35

mg/kg 3-MPA reduced the abstinence score in the last 5 min observation period, while no effect was obtained in the other observation periods. Pretreatment with 25 mg/kg did not reduce abstinence behaviour in any observation period.

3.7.4. The effect of 3-mercaptopropionate on DPA-induced motor activity

Though the suppressive effect of DPA-induced abstinence behaviour was only moderate, more pronounced effects were observed on motor activity parameters. The results in Fig. 3.7.3 show that all doses were equally effective in reducing total activity. Both horizontal and vertical activity were reduced after 25 mg/kg

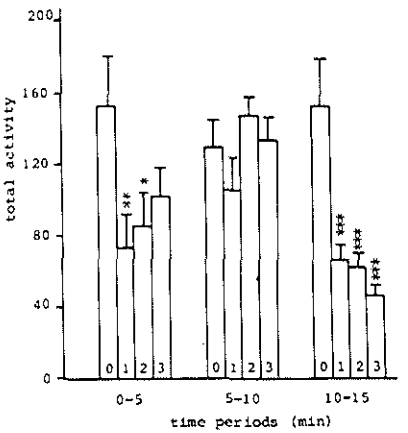


Fig. 3.7.3. Effect of 3-MPA on DPA induced motor activity. The experimental conditions are as described in the legend of Fig. 3.7.2. Activity was measured during 15 min after DPA administration. Experimental points: total activity (–o–o–), vertical activity (–•–•–) and horizontal activity (–△–△–). Statistics (Mann Whitney U-test, one tailed):
*: $p < 0.05$; **: $p < 0.02$; ***: $p < 0.003$, when compared with pretreatment with saline.

3-MPA, while higher doses seemed to be less effective. The effect of 3-MPA on total activity during sub-periods of 5 min is shown in Fig. 3.7.4. All three doses decreased total activity after DPA administration in the third 5 min sub-period, while no significant reduction was observed during the second period. During the first 5 min sub-period only 25 and 30 mg/kg 3-MPA significantly reduced total activity. Comparable results were obtained with horizontal and vertical activity. Horizontal and vertical activity were both reduced in the third 5 min observation

period when compared with saline; no effect of 3-MPA was observed during the second 5 min observation period. During the first 5 min period after administration of DPA horizontal activity was only reduced by 25 mg/kg 3-MPA, while vertical activity was reduced by all treatments. It can be concluded that 3-MPA suppressed DPA-induced motor activity parameters during the first and third 5 min observation period, whereas these parameters were not affected in the second 5 min period, when abstinence behaviour is most intense.

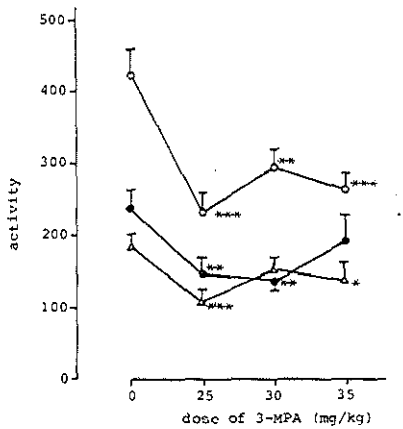


Fig. 3.7.4. Effect of 3-MPA on the time course of DPA induced total activity. The experimental conditions are as decribed in the legend of Fig. 3.7.2. The bars refer to the mean score per animal ± S.E. The treatments were saline (0), 25 (1), 30 (2) or 35 mg/kg 3-MPA. Statistics (Mann Whitney U-test, one tailed):
*: $p < 0.05$; **: $p < 0.02$; ***: $p < 0.002$.

3.7.5. Discussion

The purpose of the experiments with TSC and 3-MPA was to study whether inhibition of GABA-synthesis via glutamate decarboxylase inhibition could prevent the DPA-induced abstinence behaviour. Previous experiments showed that DPA caused an increased concentration of GABA at receptor sites suggesting that the accumulated GABA was released into the synaptic cleft. Experiments with numerous glutamate decarboxylase inhibitors have shown that inhibition of glutamate decarboxylase to a level below 60% of control levels invariably induces convulsions as the result of a decreased release of GABA from nerve endings (TAPIA, 1975). Therefore it was expected that glutamate decarboxylase inhibitors might antagonize the DPA-induced increase of GABA

at receptor sites.
The experiments with TSC indicate that moderate inhibition of glutamate decarboxylase activity cannot antagonize the DPA-induced abstinence behaviour, presumably because the inhibition was only moderate and probably less than 40% since no convulsions were observed during the observation periods. When considering a dynamic model for synthesis and degradation of GABA, these results indicate that GABA accumulation may still be effective in increasing GABA release. This means that net accumulation and subsequent release of GABA still occurred in TSC-pretreated rats. A possible explanation might be that the inhibition of glutamate decarboxylase by TSC did not affect the compartment were GABA is accumulated as the result of SSA-dehydrogenase inhibition by DPA. However, accumulation of GABA can only occur with a concurrent and relatively unimpaired synthesis accompanying the inhibition of GABA degradation. The only conclusion left is therefore that the accumulation of GABA is such that an incomplete inhibition of GABA synthesis does not prevent GABA accumulation sufficiently to inhibit the subsequent release of GABA. Since there is still an unimpaired release of GABA causing abstinence behaviour, these results suggest that the remaining synthesizing capacity after incomplete inhibition of glutamate decarboxylase by TSC is still sufficient to result in accumulation and release of GABA.
The experiments with 3-MPA revealed an inhibition of abstinence behaviour in the third 5 min observation period, while most motor activity parameters were affected with all three doses of 3-MPA in the first and third 5 min observation period. KARLSSON et al. (1974) suggested that glutamate decarboxylase activity was completely inhibited by the treatment of 35 mg/kg 3-MPA or more, because the convulsion time was no longer influenced by the dose, and because glutamate decarboxylase was inhibited for 50% in brain homogenates despite the five fold dilution of the drug, which was administered *in vivo*. By using this dose of 3-MPA inhibition of DPA-induced abstinence behaviour was observed during the third 5 min observation period. The use of these convulsant doses of 3-MPA was only possible because DPA provided a complete protection against 3-MPA-induced running fits. It might be that more delay between the administration of 3-MPA and DPA would result in a more pronounced effect. However, such an injection schedule cannot be realized very easily since running fits are produced within 6 min after administration of 3-MPA. 3-MPA may have an inhibitory effect on GABA-transaminase which might be responsible for the observed inhibition of DPA-induced abstinence behaviour as described for AOAA in the foregoing chapter. However, there is no reason to believe that GABA-transaminase is inhibited substantially *in vivo* (KARLSSON et al., 1974), as the main effect of 3-MPA is producing convulsions associated with a decrease of GABA. Therefore the predominant effect of 3-MPA is inhibition of glutamate decarboxylase *in vivo*.

In conclusion these experiments demonstrate that at least convulsant doses of glutamate decarboxylase inhibitors are required to suppress DPA-induced abstinence behaviour, suggesting that GABA metabolism is almost completely inhibited by DPA.

3.8. ANTI-CONVULSANT AND CONVULSIONS-STIMULATING PROPERTIES OF DPA

DPA has been shown to have anti-convulsant properties in a number of experimental models including models for partial seizures with elementary or complex symptomatography, absence seizures and generalized tonic-clonic seizures (SIMON & PENRY, 1975). Its anti-convulsant activity paralleled the increase of GABA (SIMLER et al., 1973; ELAZAR & GOTTESFELD, 1975; CIESIELSKI et al., 1975) and the penetration of labellet DPA (CIESIELSKI et al., 1975) into the brain. Using 300 mg/kg DPA in mice sensitive to audiogenical stimuli protection against seizures was afforded within 2.5 min after administration, while it returned to control levels after about 120 min (SIMLER et al., 1973). However, others were not able to demonstrate a correlation between the increase of GABA in the brain and the anticonvulsant properties of DPA (ANLEZARK et al., 1976). They suggest that changes in other neurotransmitter systems as a consequence of the increase of GABA after DPA administration might occur, which might be responsible for the anti-convulsant properties of DPA. Several aspects of GABA-mediated inhibition and its relation with epilepsy have recently been reviewed by MELDRUM (1975). In this chapter the effect of the various treatments modifying the GABA-ergic system and producing convulsions and its modification by DPA administration will be briefly discussed.

3.8.1. Experimental

The effect of DPA on bicuculline-induced convulsions was investigated in a separate study. Groups of 12 rats were injected with 300 mg/kg DPA or saline and received 1,2 or 3 mg/kg bicuculline 5 min later. The rats were observed for another 15 min and the incidence of convulsions and its onset, the time between administration of bicuculline and the first convulsion, were noted. For the experiments with picrotoxine the results were obtained during the abstinence experiments (chapter 3.5), during which both the incidence, onset after picrotoxine administration as well as the duration were noted. In these experiments rats were pretreated with 5 mg/kg picrotoxine and received 300 mg/kg DPA or saline after 10 min. The observations were continued for another 30 min.

The experiments with the glutamate decarboxylase inhibitors refer to the experiment where 25 mg/kg thiosemicarbazide was administered to 2 groups of rats. One group of 13 rats received an injection with 300 mg/kg DPA, while a second group of 5 rats was treated with saline (see also chapter 3.7). The rats were checked every 5 min after the administration of DPA for the presence of convulsions and death, from which the time of onset of convulsions could be

found. The experiments with 3-MPA using convulsant doses of this glutamate decarboxylase inhibitor were only done after pretreatment with DPA, which offered a complete protection. In a separate study rats were treated with varying doses of 3-MPA and saline 1 min later to demonstrate the anti-convulsant action of DPA administered 1 min after 3-MPA.

3.8.2. The effect of DPA on bicuculline-induced clonic convulsions

Initial experiments using varying doses of bicuculline indicated that the incidence of convulsions was increased after treatment with 300 mg/kg DPA. Therefore, the effect of DPA on bicuculline-induced convulsions was studied. The results in Table 3.8.1 indicate that DPA administration decreased the time of onset for the convulsant dose of 3 mg/kg bicuculline.

Table 3.8.1.

Effect of DPA on bicuculline induced clonic convulsions

Bicuculline (mg/kg)	number of convulsing rats		time of onset (sec)	
	saline	DPA	saline	DPA
1	0/12	1/11	–	114
2	2/12	4/12	(65; 235)	72.5 ± 8.3
3	6/12	9/12	90.5 ± 13.3	59.1 ± 5.7*

Groups of 12 rats were injected with saline or 300 mg/kg DPA and 2 min later 1, 2 or 3 mg/kg bicuculline (dissolved in saline at pH 3.5) was administered. The rats were observed for 5 min and the incidence of convulsions and the time of onset was noted. Statistics (Mann Whitney U-test, two tailed):
*: $p < 0.02$, when compared with the saline pretreated group (3 mg/kg bicuculline treatment).

3.8.3. The effect of DPA on picrotoxine-induced convulsions

For the experiments with abstinence behaviour a convulsant dose of DPA was used to obtain a pronounced suppression of DPA-induced abstinence behaviour (see chapter 3.5). The convulsant activity of picrotoxine was moderate and consisted of myoclonic head-twitches during about 10 sec when picrotoxine was administered alone. The results in Table 3.8.2 show that there was a tendency for all parameters tested to be increased after administration of DPA. Due to the relatively small number of animals in every group studied the effects did not reach significance.

Table 3.8.2.

Effect of DPA on picrotoxine induced convulsions

Parameter	Treatment	
	PIC x SAL	PIC x DPA
Number of convulsing rats	5/8	7/8
Onset to convulsions (min)	16.1 ± 0.9	13.9 ± 0.5*
Duration (sec)	10	20 - 30

Experimental details are exactly as described in the legend of Fig. 3.5.1.; the incidence of convulsions, duration and the time of onset was noted. Statistics (Mann Whitney U-test, two-tailed):
*: $p < 0.10$, when compared with the saline pretreated group.

3.8.4. The effect of DPA on thiosemicarbazide-induced convulsions

After administration of 25 mg/kg thiosemicarbazide convulsions developed in all rats with a time of onset of 78.0 ± 7.7 (5) min, which were all fatal for the animals. Treatment with DPA (300 mg/kg) 1 min after the administration of TSC did not afford protection against the fatal effect of TSC. However, the time of onset for the development of convulsions was significantly delayed.

Table 3.8.3.

The effect of DPA on TSC induced convulsions

Parameter	Treatment	
	TSC x SAL	TSC x DPA
Number of convulsing rats	5/5	12/12
Incidence of deaths	5/5	12/12
Onset to convulsion (min)	78.0 ± 7.8	106.7 ± 6.7*

One group of 5 rats was pretreated with 25 mg/kg TSC and saline 1 min later. A second group of 12 rats was pretreated with 25 mg/kg TSC and 300 mg/kg DPA 1 min later. The animals were checked every 5 min to note the presence of convulsions and deaths. Statistics (Mann Whitney U-test, one tailed):
*: $p < 0.01$, when compared with the saline treated group.

3.8.5. The effect of DPA on 3-mercaptopropionate-induced convulsions

Administration of 25, 30 or 35 mg/kg 3-MPA resulted in running fits, followed by convulsions in most of the animals with a short latency. For the experiments

described in chapter 3.7 advantage was taken from the strong protective effect of DPA against these 3-MPA-induced running fits and convulsions. As a consequence convulsant doses of 3-MPA could be used to demonstrate its effect on DPA-induced abstinence. The treatment with 300 mg/kg DPA afforded complete protection against these running fits and convulsions. In a parallel experiment the convulsant effect of these doses of 3-MPA was demonstrated, as is shown in Table 3.8.4. Those rats that did not have running fits and convulsions, suffered from myoclonic head-twitches except 2 rats receiving 25 mg/kg 3-MPA.

Table 3.8.4.

The effect of DPA on 3-MPA induced running fits

Pretreatment mg/kg 3-MPA)	Treatment	
	3-MPA x SAL	3-MPA x DPA
25	1/6†	0/7
30	5/6	0/7*
35	6/6	0/7**

Three groups of 6 rats were pretreated with 25, 30 or 35 mg/kg 3-MPA and received saline after 1 min. Another three groups of 7 rats were pretreated with similar doses of 3-MPA, but were injected with 300 mg/kg DPA after 1 min. The incidence of running fits and convulsions was noted. Statistics (Fisher's exact probability test, one tailed):
*: $p < 0.025$; **: $p < 0.005$, when compared with saline treated groups †: 3 additional rats suffered from myoclonic head-twitches, giving 4/6 suffering from convulsions. ($p < 0.05$, when compared with the 3-MPA x DPA treated group).

3.8.6. Discussion

Though the main action of DPA in the brain is probably inhibition of GABA-degradation, a slight inhibitory effect on glutamate decarboxylase might also be possible. As observed *in vitro*, high concentrations of DPA may inhibit glutamate decarboxylase. The enzyme was inhibited with 22.6% in the presence of 50 mM DPA, used as the sodium salt (GODIN et al., 1969). This inhibition might be caused by the inhibitory effect of Na^+ ions on glutamate decarboxylase activity measured in the presence of a K^+-containing buffer (ROBERTS & SIMONSEN, 1963). Moreover, later studies indicated that the maximal concentration of DPA in certain extrapyramidal brain structures (globus pallidus, caudate-putamen), body of fornix or commisura anterior was between 50 and 135 mM, suggesting that some inhibition of glutamate decarboxylase might occur with DPA-administration. However, because GABA degradation is much more sensitive for DPA (GODIN et al., 1969; HARVEY et al., 1973; ANLEZARK et al., 1976) the predominant action of DPA will be inhibition of GABA degradation. However, the

observed potentiating effect of DPA on bicuculline and possibly also picrotoxine induced convulsions observed in this study might be explained by an inhibition of glutamate decarboxylase *in vivo*. Inhibition of glutamate decarboxylase activity will potentiate the occurrance of convulsions, independent of the GABA-concentration, by its own (TAPIA, 1975). However, this possible inhibitory effect of DPA on glutamate decarboxylase activity cannot explain the potentiating effect on bicuculline and picrotoxine induced convulsions because DPA provided an excellent protection against the convulsions produced by 3-MPA as demonstrated in Table 3.8.4.

This paradoxical potentiation by the anti-convulsant DPA might be explained by the observation of STACH & KACZ (1976) that injection of GABA into the caudate nucleus of the rabbit resulted in a potentiation of epileptiform activity induced by epicortical administration of ouabain. This potentiation was observed immediately after administration of GABA, lasted about 15 min and was followed by an inhibition between 45 and at least 120 min after administration. Similar injections of dopamine or haloperidol resulted in inhibition or potentiation of epileptiform activity, respectively. It was concluded by the authors that the initial potentiating effect of GABA on ouabain induced epileptiform activity might be caused by an inhibition of the release of dopamine by GABA, either directly in the nucleus caudatus, or indirectly via the GABA-ergic feed-back loop inhibiting dopamine release at the level of the substantia nigra. Therefore, the initial potentiating effect of DPA on bicuculline and possibly also picrotoxine induced convulsions might be caused by an inhibition of dopamine release in the nucleus caudatus. The high level of DPA recovered in this area after administration of 400 mg/kg DPA (CIESIELSKI et al., 1975) suggests that DPA may have a predominant effect in this brain area.

Additional support for an inhibition of dopamine release in the extrapyramidal nuclei is the observation that administration of DPA results in catalepsy in a significant portion of the treated rats between 15 and 30 min after its administration (see chapter 3.9). Catalepsy observed after administration of neuroleptics has been associated with an inhibition of dopaminergic transmission in the caudate-putamen (KEHR et al., 1972; FOG, 1972; COSTALL & NAYLOR, 1972; KUSCHINSKY & HORNYKIEWICZ, 1972; EZRIN-WATERS et al., 1976).

The remarkable protection afforded by DPA against 3-MPA induced convulsions and running fits observed in the present study can be simply explained by a direct effect of DPA at the site where 3-MPA probably causes convulsions, viz. the GABA-ergic terminals where glutamate decarboxylase is largely located (see chapter 1.4.2). This observation clearly indicates that DPA inhibited GABA-metabolism in nerve endings protecting the animal against the inhibition of GABA release after administration of 3-MPA. This finding strongly supports our working hypothesis that DPA affects GABA degradation in GABA-ergic nerve

terminals where it may compensate for an inhibition of GABA release after 3-MPA administration or where it may cause an increase of GABA release as suggested from the experiments described in the foregoing chapters.
The delayed onset of TSC-induced convulsions observed after pretreatment with DPA cannot be explained with the same mechanism. The time course of DPA-induced abstinence behaviour suggests that the protection by DPA may be only short lasting. This delayed onset of TSC-induced convulsions is therefore probably associated with the increase of GABA concentration and the concurrent anti-convulsant action (SIMLER et al., 1973; ELAZAR & GOTTESFELD, 1975; CIESIELSKI et al., 1975). These considerations suggest that the proposed anti-convulsant effect of DPA might be caused by two phenomena: Firstly, the increase of GABA in nerve terminals may provide protection against the convulsant action of inhibitors of GABA synthesis like 3-MPA; and secondly, the increase of GABA which is probably associated with an inhibition of GABA degradation in glial cells and provided an anti-convulsant action of its own, as demonstrated here with the convulsant action of TSC.

3.9. DPA-INDUCED CATALEPSY

GABA may inhibit the release of dopamine from dopaminergic neurons at the level of its cellbody in the substantia nigra or at the level of the dopaminergic terminals in the caudate-putamen (BARTHOLINI & STADLER, 1975). Therefore, the rats used for the measurement of DPA-induced abstinence behaviour were routinely checked for the presence of catalepsy. In this chapter the data for the pharmacological effects on DPA-induced abstinence will be compared with the effect on DPA-induced catalepsy.

3.9.1. Experimental

For the measurement of catalepsy the rats were placed on a 7 cm high bar with their forepaws according to KUSCHINSKY & HORNYKIEWICZ (1972). The rats were considered cataleptic if they remained in that position for more than 15 sec. All rats were injected with 300 mg/kg DPA after or before several treatments. Treatment with saline or AOAA, TSC, bicuculline, picrotoxine, strychnine, 3-MPA or morphine alone never resulted in catalepsy in the present test.

3.9.2. DPA-induced catalepsy using varying amounts of DPA

Using 50 to 400 mg/kg DPA, no catalepsy was observed with saline or with 50 mg/kg DPA. Only the treatment with 300 mg/kg DPA resulted in a significant catalepsy (4/6 compared with 0/6 for saline treated rats, Fisher's test, one tailed: $p < 0.05$), whereas with 150 or 400 mg/kg DPA only 1/6 or 3/6 were cataleptic, respectively. Using a large number of animals treated with 300 mg/kg DPA in the various experiments summarized in Table 3.9.1. 60% of the treated rats were cataleptic (58/96), while no catalepsy was observed in the 96 saline treated controls.

3.9.3. Effect of GABA-ergic drugs and morphine on DPA-induced catalepsy

Potentiation of DPA-induced catalepsy by morphine. Initially, doses of morphine up to 10 mg/kg were used to suppress DPA-induced abstinence behaviour. This resulted in loss of righting reflex and the dose of morphine had to be reduced to 1 or 2 mg/kg to avoid this strong sedative effect. The results in Table 3.9.1 demonstrate that 1 mg/kg morphine did not affect the catalepsy induced by DPA administration. However, increasing the dose to 2 mg/kg morphine not only reduced the motor activity parameters as demonstrated in chapter 3.3,

Table 3.3.1, but also potentiated the DPA-induced catalepsy. Loss of righting reflex was observed for 3/12 rats 10 min after morphine administration to DPA-pretreated rats using 2 mg/kg morphine. A tendency towards a longer duration of the catalepsy after this treatment with 2 mg/kg morphine was noted. Moreover, 3/12 rats had lost their righting reflex after the combined treatment with 300 mg/kg DPA and 2 mg/kg morphine, while no loss of righting reflex was observed in rats treated with DPA alone.

Table 3.9.1.

The effect of various treatments on DPA induced catalepsy

Treatment	dosage (mg/kg)	(N)	number of rats cataleptic after treatment with		effect on DPA abstinence
			saline	test-drug	
bicuculline	2	16	7	7	reduced
morphine	1	12	4	3	reduced
morphine	2	12	4	11**	blocked
AOAA	6.3	6	3	3	unaffected
AOAA	9.4	6	3	1	unaffected
AOAA	12.5	12	6	1*	reduced
AOAA	15.7	6	3	1	blocked
3-MPA	25	7	5	6	unaffected
3-MPA	30	7	5	6	reduced
3-MPA	35	7	5	5	reduced
TSC	25	13	11	10	unaffected
strychnine	0.9	5	2	0	unaffected

The following injection schedules were used: AOAA was administered 20 min before DPA; TSC and 3-MPA were administered 1 min before DPA; bicuculline, morphine and strychnine were administered 5 min after DPA. In all cases catalepsy was measured 15 min after the administration of 300 mg/kg DPA. Statistics (Fisher's exact probability test):
*: $p < 0.05$, one tailed, when compared with saline; **: $p < 0.02$, two tailed, when compared with saline.

Suppression of DPA-induced catalepsy by AOAA. Pretreatment with AOAA resulted in a suppression of DPA-induced catalepsy by doses higher than 6.3 mg/kg. This reduction was significant for the rats treated with 12.5 mg/kg AOAA. No signicant effect was observed after the other treatments, probably because the groups were too small to reach statistical significance.
Lack of effect on DPA-induced catalepsy by bicuculline and 3-MPA. Although both drugs decrease DPA-induced abstinence behaviour significantly, no effect was observed on DPA-induced catalepsy. Treatment with TSC and strychnine were without effect on abstinence behaviour as well as catalepsy induced by DPA.

3.9.3. Discussion

A strio-nigral pathway has been described exerting an inhibitory action on dopaminergic cellbodies located in the substantia nigra (for references and a review of the available evidence, see chapter 1.3.2.6). The existance of such a GABA-dopaminergic interaction is supported by the observation that intranigral injection of GABA or AOAA may reduce the turnover of dopamine in striatum (ANDEN & STOCK, 1973; ANDEN, 1974). Further support is provided by the enhanced release of dopamine from the surface of the caudate nucleus after infusion of the GABA antagonist bicuculline into substantia nigra (BARTHOLINI & STADLER, 1975). Moreover, the presence of a GABA-ergic system exerting an inhibitory influence on dopaminergic terminals within the nucleus caudatus was demonstrated by the intracaudal injection of picrotoxine and bicuculline or GABA, which increased or decreased the release of dopamine, respectively (BARTHOLINI & STADLER, 1975). These results suggest that the GABA-ergic system is involved in the control of dopaminergic transmission on two levels: the substantia nigra, where GABA exerts an inhibitory action on dopaminergic cellbodies, and the caudate nucleus where GABA-ergic interneurons may exert an inhibitory action on dopaminergic terminals. The observed antagonism by AOAA of neuroleptic-induced increase of dopamine turnover (LAHTI & LOSEY, 1974), the potentiation by AOAA of the cataleptogenic action of neuroleptics (HAEFELY et al., 1975) and the antagonism by intranigral injection of the GABA agonist muscimol of the neuroleptic-induced increase of the affinity of tyrosine-hydroxylase for the pteridine-cofactor observed by GALE & GUIDOTTI (1976) are in accordance with this GABA-ergic control of dopaminergic function in the extrapyramidal system.

The present experiments demonstrating that an increased release of GABA may result in catalepsy in a significant portion of the treated animals suggests an inhibitory GABA-ergic influence on extrapyramidal dopaminergic structures; because catalepsy is considered as a reliable behavioural correlate of an impaired dopaminergic function (KEHR et al., 1972; FOG, 1972; COSTALL & NAYLOR, 1972; KUSCHINSKY & HORNYKIEWICZ, 1972; EZRIN-WATERS et al., 1976). This result is in accordance with findings of others summarized above demonstrating that dopaminergic transmission is under the inhibitory influence of the GABA-ergic system. The results in Table 2.9.2 suggest that the catalepsy is only transient being present between 15 and 30 min after DPA-administration. Whether catalepsy is present shortly after DPA has not been studied. An inhibition of dopamine release has also been suggested to occur during precipitated morphine abstinence (IWAMOTO et al., 1973 and 1976; COX et al., 1976) demonstrating the close relationship between DPA-induced and morphine abstinence behaviour.

Morphine and AOAA both suppressed DPA-induced abstinence behaviour,

whereas only AOAA also suppressed the DPA-induced catalepsy. This suggests that the DPA-induced abstinence behaviour and catalepsy arise from the same phenomenon, which can be suppressed by AOAA. The potentiation by morphine of DPA-induced catalepsy might be caused by the sedative effect of this dose of morphine which reduced horizontal activity considerably below the level in saline treated controls. Morphine itself may also give catalepsy, but this phenomenon is only induced with much higher doses of morphine (KUSCHINSKY & HORNYKIEWICZ, 1972). Important in this respect is that no potentiation of DPA-induced catalepsy was observed with 1 mg/kg or less (not shown), indicating that this potentiating effect by morphine on DPA-induced catalepsy is not related to the suppressive effect of morphine on DPA-induced abstinence behaviour. It is suggested that morphine may potentiate the DPA-induced suppression of locomotor activity observed by others after treatment with DPA (MISSLIN et al., 1976).

When comparing the effect of AOAA and DPA it is worthwhile to mention that AOAA itself does not cause catalepsy, although it may potentiate the cataleptogenic action of neuroleptics (HAEFELY et al., 1975). Nevertheless, AOAA and DPA seem to have opposite effects as demonstrated by the inhibition by AOAA of DPA-induced abstinence behaviour and DPA-induced catalepsy. This suggests that both inhibitors of GABA degradation have opposite effects in the GABAergic system.

In conclusion, these experiments demonstrating that DPA administration results in catalepsy suggests that DPA-induced GABA release may inhibit dopaminergic transmission. Although the effect of DPA is only transient it is worthwhile to compare the action of neuroleptics with that of DPA.

4. GENERAL DISCUSSION

4.1. POSSIBLE REGULATORY FUNCTION OF SSA-DEHYDROGENASE IN GABA-DEGRADATION

A regulatory function of SSA-dehydrogenase in the degradation of GABA has been ignored for the following reasons. The product of GABA degradation, SSA, has a high affinity for SSA-dehydrogenase (EMBREE & ALBERS, 1964; KAMMERAAT & VELDSTRA, 1968) and cannot be detected in the brain. This suggests that SSA is converted into succinate as soon as it is formed. The finding that SSA-dehydrogenase is about twice as active as GABA-transaminase (PITTS et al., 1965; this thesis, chapter 2.3.1.) also suggests that GABA-transaminase is rate-limiting in GABA degradation, whereas SSA-dehydrogenase is not.

In the biochemical section of this thesis a method is introduced for measuring GABA-transaminase. This method takes advantage of two properties of SSA-dehydrogenase; the high affinity of SSA for the dehydrogenase and the twofold excess of SSA-dehydrogenase over GABA-transaminase. These two properties allow the measurement of GABA-transaminase activity via a coupled enzyme method using the endogenously present dehydrogenase to convert all SSA formed further into succinate. Measurement of the kinetic parameters of SSA-dehydrogenase from time courses at various concentrations of SSA revealed that the affinity of SSA for SSA-dehydrogenase was rather low giving a K_m of 138 μM at a tissue concentration of 2.22 mg/ml. The calculated V_{max} of 64.5 μmol/g/h is in accordance with the literature, indicating that SSA-dehydrogenase was about twice as active as GABA-transaminase in homogenates (SHERIDAN et al., 1967; MILLER & PITTS, 1967). This low affinity of SSA for SSA-dehydrogenase under the conditions of the GABA-transaminase assay indicated that the condition of a high affinity of SSA for SSA-dehydrogenase, a prerequisite for a reliable GABA-transaminase assay, was not fulfilled. However, despite this low affinity of SSA for the dehydrogenase, the GABA-transaminase assay was found reliable, because similar results were obtained with a radiochemical method (where both SSA and succinate were measured) and with the coupled enzyme method (where only NADH was measured). A further study revealed that the affinity of SSA for the dehydrogenase was dependent on tissue concentration. It was concluded that a high and low affinity form of SSA-dehydrogenase might be present in brain homogenates. The high affinity form of SSA-dehydrogenase apparently prevented the SSA from accumulation in the GABA-transaminase assay.

The experiments described in chapter 3.7 indicate that SSA might also have a high affinity for GABA-transaminase. It was expected that the measurement of GABA-transaminase using the radiochemical method would give similar results

when measured with or without NAD, i.e. with and without an active coupling of SSA-dehydrogenase to remove all SSA converting it into succinate. However, just the opposite was found, indicating that the backward reaction of GABA-transaminase was active in the presence of low concentrations of SSA and glutamate.

These *in vitro* studies suggested that SSA might have a selective affinity for GABA-transaminase or SSA-dehydrogenase, respectively, depending on several factors such as: the concentration of glutamate, the affinity of SSA for SSA-dehydrogenase, the presence of Na^+ etc. This suggests that SSA-dehydrogenase might have a regulatory function in the control of GABA degradation under physiological conditions as described in chapter 2.7. This possibility has always been ignored on the base of *in vitro* studies which indicate that the affinity of SSA for SSA-dehydrogenase is high. Our observation that under certain experimental conditions this may not be the case reopens this discussion.

A further study of *pseudomonas* SSA-dehydrogenase demonstrated the presence of two forms of SSA-dehydrogenase with a high or a low affinity, respectively. Both were activated to the same extent by Na^+ suggesting that they represent two froms of the same enzyme. This is in accordance with the observation that two forms of SSA-dehydrogenase were present in rat brain homogenate. The experiments with varying concentrations of monovalent or bivalent cations indicated that SSA-dehydrogenase was activated by monovalent cations in close relationship with their ionic radius. 25 - 100 mM Na^+ activated SSA-dehydrogenase by 75%, a value similar to that observed for the rat brain enzyme using 50 - 100 mM Na^+. An increase by Na^+ of V_{max} was observed for the rat brain as well as for the *pseudomonas* enzyme, whereas no activation of GABA-transaminase of rat brain by monovalent cations occurred. The observations that Ca^{2+} may activate or inhibit SSA-dehydrogenase depending on whether SSA was administered before or after the Ca^{2+}, suggests a regulatory function of Ca^{2+} for the *pseudomonas* enzyme. It remains to be established whether these results for Ca^{2+} can be extended to the rat brain enzyme.

These results demonstrate that *pseudomonas* and rat brain SSA-dehydrogenase have many properties in common. Therefore, it is suggested that *pseudomonas* SSA-dehydrogenase might be useful as a model for the complicated rat brain enzyme. The selective effect of monovalent and bivalent cations on the activity and on the properties of SSA-dehydrogenase indicates that its activity might be susceptible to change in the ionic environment *in vivo*. Such changes are generally associated with physiological processes such as neurotransmitter release or cell-depolarization. Therefore, these findings provide additional support for a regulatory function of SSA-dehydrogenase in the control of GABA degradation.

4.2. DIFFERENTIAL EFFECTS OF GABA ANALOGUES ON THE GABA-SHUNT ENZYMES

The purpose of these experiments was to investigate whether the dyskinesias observed after administration of zinc to mice or other species (BONTA et al., 1964; BONTA, personal communication) could be related to inhibition of one of the GABA shunt enzymes by zinc. The antagonism observed after administration of comparably low doses of HA-966 of these zinc induced dyskinesias might arise from the metalion complexing properties of HA-966 which was the starting point of those zinc studies (BONTA et al., 1964). The experiments with glutamate decarboxylase, GABA-transaminase and SSA-dehydrogenase indicate that the pharmacological effects of zinc and HA-966 might arise from their opposite effects on the enzymes of the GABA-shunt. Although zinc may inhibit glutamate decarboxylase *in vitro* in low concentration, it may activate PLP-kinase considerably (TWOMEY & BAXTER, 1973). This suggests that zinc administration may increase the concentration of GABA *in vivo*, due to its predominant inhibitory effect on the GABA degrading enzymes. The inhibition pattern of NH-DABA was similar to that of zinc, although PLP is probably involved in the inhibitory effect of NH-DABA as opposed to the effect of zinc. Again, the net effect depends partially on PLP-kinase which may form sufficient PLP *in vivo* to compensate for the complex-formation of PLP with NH-DABA. Therefore, it was concluded that the quasi-morphine abstinence behaviour observed after intraventricular administration of zinc or NH-DABA was associated with an increase of GABA as the result of an inhibition by these compounds of GABA-degradation.

The only compound having specific effects on the GABA-shunt enzymes is p-HBA. It inhibited SSA-dehydrogenase and possibly GABA-transaminase, but had no effect on glutamate decarboxylase activity. 2,4-diaminobutyrate, on the other hand, inhibited glutamate decarboxylase and GABA-transaminase, but was without effect on SSA-dehydrogenase. This suggests that selectivity for drugs inhibiting the GABA-shunt enzymes can be obtained by comparing the effects of drugs on glutamate decarboxylase and SSA-dehydrogenase, while neglecting the effect on GABA-transaminase. Apparently, the structural requirements for inhibition of SSA-dehydrogenase differ from those required for an inhibition of glutamate decarboxylase, whereas GABA-transaminase has an intermediate position. The presence or absence of a substituent on the α-carbon might be important in this respect.

4.3. DPA-INDUCED ABSTINENCE BEHAVIOUR AS A CHARACTERISTIC OF INCREASED GABA-ERGIC ACTIVITY *IN VIVO*

The GABA-ergic nature of the DPA-induced abstinence behaviour has been demonstrated by using the two selective GABA antagonists picrotoxine and bicuculline. Both drugs inhibited the abstinence behaviour and the associated changes in motor activity parameters, while the catalepsy observed after 15 min, was not affected by these treatments. The selectivity of the effect of the GABA-antagonists was further demonstrated by the ineffectiveness of strychnine using threshold doses for inducing convulsions. Strychnine had no effect on the DPA induced abstinence behaviour, and the observed mild myoclonic head-twitches apparently did not interfere with the DPA-induced abstinence behaviour. This indicates that the antagonism of the DPA-induced abstinence behaviour was caused by the GABA blocking properties of picrotoxine and bicuculline, whereas the convulsant properties as such were not responsible for the observed suppressive effect of these compounds. The inhibition of GABA degradation *in vitro* and the increase of GABA observed by some authors (SIMLER et al., 1973; ELAZAR & GOTTESFELD, 1975; CIESIELSKI et al., 1975) provide additional evidence for the GABA-ergic nature of this DPA-induced abstinence behaviour. Recent evidence suggests that DPA is a selective inhibitor of SSA-dehydrogenase being devoid of an effect on GABA-transaminase (HARVEY et al, 1975; ANLE-ZARK et al., 1976). The effect on GABA-transaminase activity observed in earlier studies (GODIN et al., 1969; FOWLER & JOHN, 1972; SIMLER et al., 1973; CIESIELSKI et al., 1975; FOWLER et al., 1975) was probably caused by inhibition of the coupling enzyme SSA-dehydrogenase, as suggested by ANLE-ZARK et al. (1976). The competitive inhibition of SSA-dehydrogenase by DPA has been confirmed in the present study (chapter 2.5) using *pseudomonas* SSA-dehydrogenase. Moreover, HARVEY et al. (1975), when measuring GABA-transaminase using SSA and glutamate as a substrate, observed that DPA did not inhibit GABA-transaminase. The observation that AOAA in low doses, probably affecting GABA-transaminase selectively, did not produce abstinence behaviour and catalepsy (chapters 3.5 and 3.9) suggests that inhibition by DPA of GABA-transaminase was not responsible for the DPA-induced abstinence behaviour. On the contrary, pretreatment with AOAA inhibited both the DPA-induced abstinence behaviour and the DPA-induced catalepsy. Although AOAA or DPA both inhibit GABA degradation and increase the concentration of GABA in the brain (VAN GELDER, 1966; GODIN et al., 1969; WOOD & PEESKER, 1973; CIESIELSKI et al., 1975; ELAZAR & GOTTESFELD, 1975), the observed behaviour is, at least initially, completely different. It is suggested that this different behaviour after AOAA or DPA is mediated by two different types of GABA-receptors: one type (I) is activated after inhibition of SSA-dehydrogenase by DPA and is involved in evoking abstinence behaviour and catalepsy (viz. activa-

tion). The second type (II) is activated after inhibition of GABA-transaminase and is associated with decreased motor activity of the rat. MISSLIN et al. (1976) observed sedation after treatment of rats or mice with varying doses of DPA. However, because the locomotor activity of the rats was measured for a period of 2 h without observing in sub-periods, an underlying initial activation of the rats during the first 15 min after administration, as observed in the present study, probably has been overlooked.

Inhibition of GABA synthesis by treatment with TSC or 3-MPA to study the effect of inhibition of glutamate decarboxylase on DPA-induced abstinence behaviour gave varying results. The administration of DPA provided a complete protection against 3-MPA induced convulsions and running fits, while the onset of TSC-induced convulsions was delayed. This suggests that the inhibition of glutamate decarboxylase, which is held responsible for these convulsions (chapter 3.6 and 3.8), was easily compensated for by the inhibition of GABA degradation by DPA. On the other hand, 3-MPA only partially antagonized the behavioural effect of DPA although convulsant doses were used. It has been suggested that the treatment with 35 mg/kg 3-MPA completely inhibits GABA-synthesis (KARLSSON et al., 1974.) The experiments with 3-MPA, which indicate that such a dose of 3-MPA only partially reduced DPA-induced abstinence behaviour and motor activity, are not necessarily in conflict with that observation. 3-MPA had to be given either shortly before or shortly after DPA to avoid convulsions. Thus accumulation of GABA had already taken place as is demonstrated by the complete protection against conculsions and running fits after DPA administration. However, a more pronounced suppressive effect of 3-MPA might be achieved by increasing the pretreatment time from 1 to about 4 min. Such a procedure might give still sufficient protection against covulsions, while the effect of 3-MPA on the GABA-ergic system is already fully developed. Nevertheless, the present results provide additional evidence for the GABA-ergic nature of the DPA-induced abstinence behaviour. The negative results obtained with TSC are probably due to the slow onset of its effect on glutamate decarboxylase and the relatively low dose of TSC used. TSC probably acts via PLP-depletion (BAXTER, 1969; TAPIA et al., 1969). Whereas complete inhibition of glutamate decarboxylase might occur with 3-MPA, continuing synthesis of PLP may maintain minimal levels of PLP and GABA synthesis in the presence of TSC.

In conclusion, these results suggest that DPA-induced abstinence behaviour is mediated via an increased GABA-ergic activity obtained by a selective inhibition of GABA degradation via SSA-dehydrogenase. Furthermore, these experiments provide *in vivo* evidence for a regulatory function of SSA-dehydrogenase in the degradation of GABA which was suspected on the basis of *in vitro* experiments described in the biochemical section of this thesis.

4.4. GABA COMPARTMENTATION AND DPA-INDUCED ABSTINENCE BEHAVIOUR

DPA-induced abstinence behaviour is associated with an increase of locomotor activity and severe abstinence signs during approximately 15 min, after which catalepsy becomes apparent. The animals become quiet and after about 30 min their behaviour was not very different from control rats. The time course of this behaviour is in striking contrast with the observed time course of the anticonvulsant activity and the closely associated increase of GABA concentration in the brain after DPA treatment (CIESIELSKI et al., 1975), which may last about 2 h. On the other hand, the fast onset of the protection against audiogenic in susceptible mice is in accordance with the almost immediate start of abstinence behaviour after administration of DPA.

Based upon the present results, two types of GABA-ergic receptors can be roughly distinguished in the rat brain: One type (I), mediating abstinence behaviour and increased locomotor activity as described in the behavioural section of this thesis; and a second type (II) mediating sedation, which is observed in association with an increase of GABA in the brain (MISSLIN et al., 1976; FREED & MICHAELIS, 1976). The type I receptors are apparently activated by SSA-dehydrogenase inhibition, while the type II receptors are activated by GABA-transaminase inhibition or application of GABA directly into the brain (FREED & MICHAELIS, 1976). The inhibition of DPA-induced abstinence behaviour by AOAA as well as the inability of AOAA to produce abstinence behaviour has been explained by an inhibitory effect of GABA, released from glial cells, on presynaptic autoreceptors in GABA-ergic terminals (see chapter 3.6, discussion). It is suggested that AOAA may inhibit the DPA-induced abstinence behaviour via an inhibition of the release of accumulated GABA from nerve terminals. A similar mechanism may terminate the DPA-induced abstinence behaviour 15 min after DPA administration. Apparently, the presynaptically located GABA-ergic (type II) receptors can also be activated by DPA as demonstrated by the observed sedation after DPA administration (MISSLIN et al., 1976). Moreover, GRIMM et al. (1975) observed that AOAA and DPA had comparable effects on the execution of learned locomotor acts requiring balancing and coordination of the hind limbs in close association with the increase in GABA concentration observed. Supposing that DPA may affect the glial as well as the neuronal pool while having its main effect on the neuronal pool, the slower onset of the GABA release from glial cells after administration of DPA may terminate the initially increased release of GABA from nerve terminals. As a result the rats become quiet and decreased locomotor activity in association with a further increase of the concentration of GABA in the brain may occur as observed by others (GRIMM et al., 1975; MISSLIN et al., 1976; FREED & MICHAELIS, 1976).

No catalepsy has been observed after treatment with AOAA, whereas AOAA administration in a dose of 12.5 mg/kg reduced the incidence of catalepsy in DPA-treated rats (chapter 3.9). Moreover, this catalepsy is only transient and notable between 15 and 30 min after DPA administration. Therefore, DPA-induced catalepsy and abstinence behaviour are caused by the same mechanism, viz. activation of GABA-ergic type I receptors. The dopaminergic character of catalepsy (KEHR et al., 1972; FOG, 192; COSTALL & NAYLOR, 1972, KUSCHINSKY & HORNYKIEWICZ, 1972; EZRIN-WATERS et al., 1976) indicates that the increased GABA-ergic activity after administration of DPA resulted in an inhibition of the release of dopamine in the extrapyramidal system, either via an inhibition at the level of cell bodies in the substantia nigra or on the dopamine terminals in striatum. As has been discussed in chapter 3.8, this inhibition of dopamine release might be held responsible for the observed potentiation of bicuculline-induced convulsions.

The working hypothesis, proposed in the present study, suggests that the AOAA induced inhibition of DPA-induced abstinence behaviour is mediated via presynaptic inhibitory GABA-ergic receptors located on GABA-ergic terminals. Additional support for this hypothesis is provided by the findings that picrotoxine alone also induced abstinence behaviour shortly after administration. This unexpected finding can be explained by the presence of presynaptic GABA-ergic receptors results in inhibition of GABA release from the nerve fibers, as is probably observed with AOAA treatment. On the other hand, blockade of these receptors results in an increase of GABA release associated with abstinence behaviour and catalepsy. A concept of GABA-ergic neurons, being under the control of presynaptically located inhibitory GABA-ergic receptors, may thus explain the unexpected abstinence behaviour of picrotoxine. Some support for a preferential effect of picrotoxine and possibly also of bicuculline is provided by electrophysiological studies demonstrating that convulsants like bicuculline or picrotoxine may potentiate the post-synaptic inhibition of GABA in many brain areas (STRAUGHAN et al., 1971; STRAUGHAN, 1974). The suggestion from these studies that presynaptic GABA-ergic receptors might be more sensitive for antagonists than the post-synaptically located ones, is in accordance with the observed abstinence behaviour after administration of picrotoxine. Such presynaptically located inhibitory GABA-ergic receptors may be sensitive to overflow of GABA from the glial compartment, where AOAA may have its predominant effect on GABA degradation. It is still uncertain whether the abrupt ending of DPA-induced abstinence behaviour is due to an increase of GABA in the glial compartment, or whether it is the result of an overflow of GABA from the synaptic region to the presynaptic receptor.

4.5. THE EFFECTS OF MORPHINE AND NALOXONE ON DPA-INDUCED ABSTINENCE BEHAVIOUR

A number of conditions have been proposed for the positive identification of quasi-morphine abstinence behaviour (see chapter 1.11). The DPA-induced abstinence behaviour, relating an overactive GABA-ergic system to morphine abstinence, seems to fulfil almost all conditions mentioned by COLLIER (1974). Firstly, it increases a number of symptoms classified as morphine abstinence symptoms, including shaking behaviour, escape digging, ptosis, hunch-back, piloerection, head and forepaw shaking and swallowing, which are rarely observed in naive rats. The absence of the peripheral signs, according to the classification made by JHAMANDAS et al. (1973), FREDERICKSON & SMITS (1973) and FREDERICKSON (1975), suggests that GABA is only involved in the expression of the so-called centrally evoked signs. Secondly, morphine in very low doses (a significant suppression was already observed after 0.2 mg/kg morphine) suppressed the DPA-induced abstinence behaviour as well as the associated increase in motor activity. This suppressive effect of morphine on DPA-induced abstinence behaviour appeared to be dissociated from the depressive effect of morphine alone on horizontal activity. This suppressive effect of morphine on DPA-induced abstinence behaviour was reversed by naloxone, while the combined treatment with morphine and naloxone was ineffective. Moreover, administration of naloxone could antagonize the suppressive effect of morphine in DPA-pretreated rats and produced abstinence behaviour at a time when DPA-induced abstinence behaviour in saline treated rats is not present anymore. Naloxone was ineffective in DPA pretreated rats, whereas naloxone has been reported to potentiate the quasi-morphine abstinence behaviour induced by phosphodiesterase inhibitors (COLLER et al., 1974; FRANCIS et al., 1975). However, a rather high dose of 10 mg/kg naloxone was used to demonstrate this potentiation of quasi-morphine abstinence behaviour by naloxone, while in the present experiments 1 mg/kg naloxone was administered. Another criterion recently suggested (COLLIER & FRANCIS, 1976) is the stereospecificity of the suppressive effects of opiates on DPA-induced abstinence behaviour.
Such studies have not been carried out yet. However, the low ED_{50} of 0.5 mg/kg morphine is very suggestive of a specific suppressive effect of morphine in DPA-induced abstinence behaviour, especially since this effect could be separated from the depressive effects on horizontal activity of morphine alone (chapter 3.4). The observations of HO et al. (1976) that GABA affects the development of tolerance and physical dependence, as suggested by the effects of bicuculline, AOAA, 2,4-diaminobutyrate and β-alanine on these phenomena, strengthens the value of the DPA-induced abstinence behaviour as a model for morphine abstinence.
As has been discussed in chapter 1.9, several experiments provided indirect evi-

dence for an action of morphine involving GABA. The effect of morphine on DPA-induced abstinence behaviour suggests that morphine in low, subanalgesic doses inhibits the release of GABA. The observed decrease of motor activity with morphine alone is in accordance with that observation as an inhibition of the release of GABA is associated with depression of motor activity. The observed potentiation of DPA-induced catalepsy by using 2 mg/kg morphine might be related to this sedative effect of morphine, although a synergistic effect of morphine on post-synaptic cells, being under the inhibitory control of GABA-ergic nerve terminals, with the increased release of GABA from nerve terminals might also be possible. The potentiating effect of morphine on the DPA-induced catalepsy clearly dissociates its mode of action from that of AOAA, which suppressed the DPA-induced catalepsy. However, both AOAA and morphine may suppress DPA-induced abstinence behaviour via presynaptic inhibition on GABA-ergic terminals. A concept of GABA-ergic nerve terminals being under the inhibitory influence of inhibitory GABA-ergic autoreceptors and the inhibi-

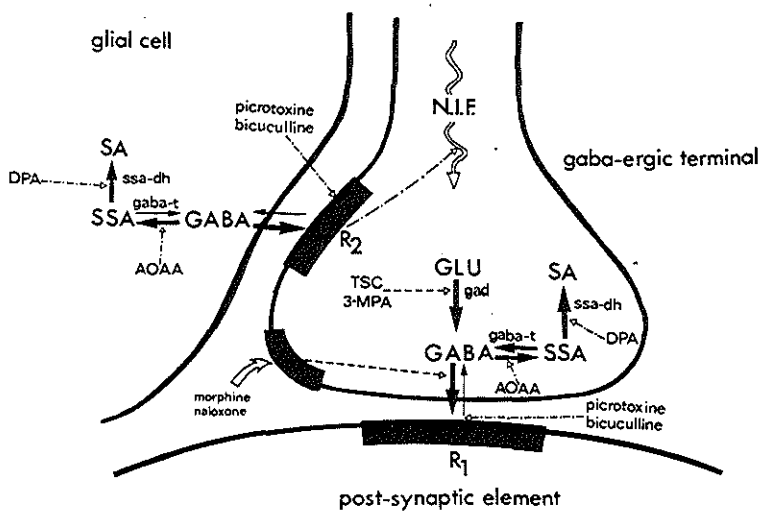


Fig. 4.2. A model of the action of morphine and GABA-ergic drugs on a GABA neuron. Two types of GABA receptors are proposed: type I, mediating abstinence behaviour and located on a post-synaptic element, and type II receptors mediating decrease of motor activity. The effect of morphine, preventing the release of GABA, might be mediated via opiate receptors located on the presynaptic GABA-ergic element. The abbreviations used for this Fig. are: GLU: glutamate; SA: succinate; GAD: glutamate decarboxylase; GABA-T: GABA-transaminase; SSA-DH: SSA-dehydrogenase; N.I.F.: nerve impulse flow; – – – –: inhibition.
a. GABA interaction with type I receptor: inhibition of post-synaptic nerve fibre (abstinence behaviour).
b. GABA-interaction with type II receptor: inhibition of GABA-release from the nerve-ending: disinhibition of the post-synaptic fibre (decrease of activity).

tory action of opiates as well, is proposed (Fig. 4.1).
The discussion presented so far tends to suggest that all pharmacological actions of morphine are mediated via the GABA-ergic system. Such a suggestion would be misleading since only some acute effects of morphine and some chronic mechanisms associated with opiate receptors have been connected to the GABA-ergic system. It must be stressed that changes in the GABA-ergic system may have secondary effects on other neurotransmitter systems, as demonstrated in the present experiments with the DPA-induced catalepsy, which is probably related to inhibition of dopaminergic transmission in striatum. Moreover, the action of morphine may be indirect, involving for example release of Ca-ion as discussed in chapter 1.10. The experiments with *pseudomonas* SSA-dehydrogenase (chapter 2.5) indicate a prominant role of Ca^{2+} in the regulation of SSA-dehydrogenase. An activation of SSA-dehydrogenase by Ca^{2+} release might occur resulting in reduction of GABA and inhibition of abstinence behaviour. Such a mechanism is, however, not very likely because SSA-dehydrogenase is a mitochondrial enzyme, where the Ca^{2+} concentration will be high.
According to the model presented in Fig. 4.1, GABA-transaminase and SSA-dehydrogenase are present in glial cells as well as nerve endings. Differential centrifugation studies indicated that appreciable amounts of GABA-transaminase and SSA-dehydrogenase are recovered in the nerve endings containing fractions (SALGANOCOFF & DE ROBERTIS, 1963 and 1965; VAN KEMPEN et al., 1965; FONNUM, 1968; SIMS & DAVIS, 1973; BUU & VAN GELDER, 1974). Moreover, recent experiments indicated that GABA-transaminase and SSA-dehydrogenase levels were only slightly lower in synaptosomes than in glial cells on a protein basis (SELLSTRÖM et al., 1975), while GABA metabolism was similar in these fractions. Therefore, it is suggested that the GABA degrading enzymes are located both in glial cells and nerve terminals (see for a further discussion, chapter 1.4). The potent anti-convulsant action of DPA towards 3-MPA and the observed partial suppression by 3-MPA of DPA-induced abstinence behaviour suggest that DPA may act preferentially in the neuronal compartment inhibiting GABA metabolism in nerve endings containing GABA. This preferential effect of DPA on the nerve ending containing GABA compartment is also based on the hypothesis that SSA-dehydrogenase might contribute to the accumulation of GABA in nerve terminals. SSA-dehydrogenase is rate limiting in this neuronal compartment, whereas GABA-transaminase might be regulatory in the glial compartment were GABA normally not accumulates.

SUMMARY

GABA is degraded by the consecutive action of two enzymes: GABA-transaminase and SSA-dehydrogenase. Inhibition of the rate limiting step in GABA-degradation may increase the functional level of GABA in the brain and might be of potential use in the treatment of neurological or psychiatric disorders. The two main objects of this thesis were therefore to find the rate-limiting step in the degradation of GABA and see whether inhibition of this enzyme by specific drugs may fascilitate GABA-ergic transmission.

In the introductory section of the thesis a survey of the biochemical pharmacology of GABA is presented, while the evidence for both presynaptic and postsynaptic inhibition mediated by GABA in several brain regions and spinal cord is discussed. The evidence for a presynaptic localization of GABA degrading enzymes is reviewed in chapter 1.4. As an increased GABA-ergic activity in the brain seems to be associated with a behavioural syndrome that can be characterized as quasi-morphine abstinence behaviour a number of aspects relevant for the experiments with morphine are discussed in chapters 1.5 till 1.11.

The activity of GABA-transaminase and SSA-dehydrogenase has been determined in total brain homogenate. GABA-transaminase activity was measured using a coupled enzyme method which utilizes endogeneous SSA-dehydrogenase to convert the formed SSA into succinate. The concurrently produced NADH was used as an estimate of GABA-transaminase activity. This method could be used since it was shown that the dehydrogenase was about twice as active as the transaminase and because no significant accumulation of the intermediate SSA could be detected. GABA-transaminase was inhibited by high ionic strength. In contrast NaCl decreased the apparent K_m and increased V_{max} for SSA-dehydrogenase. Increasing tissue concentration also resulted in a decrease of the apparent K_m, but dit not change the V_{max} of SSA-dehydrogenase and it is suggested that this enzyme can exist in two distinct states of aggregation, one with a high and one with a low affinity for SSA. The high affinity form of the enzyme is thought to prevent SSA from accumulation in the GABA-transaminase assay. It is concluded that this GABA-transaminase assay can be used within certain limits for the assay of GABA-transaminase activity.

Using *pseudonomas* SSA-dehydrogenase as a model for the rat brain enzyme it was found that the enzyme was activated considerably by addition of monovalent or bivalent cations. Optimal activations were observed with 25 to 100 mM NaCl. Using Li^+, Na^+, K^+, Rb^+, Cs^+ or NH_4^+, it was shown that monovalent cations activated the enzyme in accordance with their ionic radius giving an optimal activation with K^+ of 175%. Using Mg^{2+}, Ca^{2+}, Sr^{2+} or Ba^{2+}, maximal activation with 70% was obtained using Ca^{2+} or Mg^{2+}. The activation by monovalent cations involved an increase of V_{max}. Activation or inhibition by Ca^{2+}

could be found by giving the substrate after or before the cation. Using this preparation the presence of different enzymatic forms with high or low affinity for substrate could be confirmed. It is concluded that *pseudomonas* SSA-dehydrogenase can be used as a model for the more complicated rat brain enzyme. A model was presented, based on a selective high affinity for GABA-transaminase or SSA-dehydrogenase, respectively, which might account for accumulation of GABA in one and immediate degradation of GABA in another compartment. Based on these *in vitro* experiments it was suggested that SSA-dehydrogenase might have a regulatory function in GABA degradation. Experiments with various compounds indicated that inhibition of SSA-dehydrogenase *in vivo* might result in a selective effect on GABA degradation alone, because the structural requirements for inhibition of SSA-dehydrogenase when compared with glutamate decarboxylase were different.

Intraperitoneal administration of DPA, a SSA-dehydrogenase inhibitor, to naive rats produced abstinence behaviour including shaking, digging, hunchback posture, piloerection and ptosis during 15 min, and increased locomotor activity considerably. This behaviour could be suppressed by the treatment with a subconvulsive dose of 2 mg/kg bicuculline or by the administration of 5 mg/kg picrotoxine, a threshold dose for convulsions, while the selective glycine-antagonist strychnine was ineffective. This indicated that the syndrome was caused by an increase of GABA at receptor sites suggesting that the inhibition of SSA-dehydrogenase by DPA *in vivo* resulted in GABA release. A comparable syndrome was not provoked by the treatment with AOAA, a GABA-transaminase inhibitor, suggesting that the effect of DPA was not caused by inhibition of this enzyme. Moreover, AOAA suppressed the DPA-induced abstinence behaviour considerably suggesting that at least two GABA-ergic systems must be present in the brain with opposite effects on the behaviour. Using TSC or 3-MPA it was demonstrated that the syndrome could be suppressed by using high doses of 3-MPA that produced convulsions in control rats. No convulsions were demonstrated in DPA treated rats after administration of 3-MPA indicating that the increase of GABA after DPA administration compensated for the decreased synthesis of GABA in the presence of 3-MPA. Based on these experiments a model was presented indicating that GABA-ergic terminals were under the inhibitory influence of presynaptically located GABA-ergic autoreceptors. These experiments with DPA demonstrated that SSA-dehydrogenase has regulatory properties in at least one GABA compartment providing *in vivo* evidence for the working hypothesis that SSA-dehydrogenase is rate-limiting in GABA degradation.

The relation of this behaviour with morphine abstinence was demonstrated by the experiments with morphine and naloxone. Using a low dose of 1 mg/kg morphine an almost immediate and complete suppression of the abstinence syndrome was obtained, while administration of naloxone after morphine could release

the syndrome again. Simultaneous treatment with morphine and naloxone or naloxone alone were without effect. Using varying doses of morphine an ED_{50} of 0.5 mg/kg was found for the effect of morphine while its action could be seperated from its depressant effect on locomotor activity in non-treated animals. These experiments suggest that morphine receptors are involved in DPA-induced abstinence behaviour and suggest also that morphine abstinence is associated with an increased release of GABA. The finding that a significant proportion of the DPA treated rats was cataleptic after 15 min suggests that other transmitter systems are under the influence of the GABA-ergic system indicating that the interrelationship of the GABA-ergic system with other systems can be studied by using this model of increased GABA-ergic activity.

SAMENVATTING

GABA wordt afgebroken door twee enzymen: GABA-transaminase en SSA-dehydrogenase. Remming van de snelheids bepalende stap in de afbraak van GABA zou het functionele niveau van GABA in de hersenen kunnen verhogen en zo van betekenis kunnen zijn voor de behandeling van bepaalde neurologische en psychiatrische ziektebeelden. De belangrijkste doelstellingen van dit proefschrift waren dan ook het vinden van de snelheids bepalende stap in de afbraak van GABA en het remmen van dit enzyme *in vivo* met specifiek werkende farmaka om zo de GABA-erge transmissie te bevorderen.
In de inleiding van dit proefschrift is een overzicht gegeven van de biochemie en farmacologie van GABA (hoofdstuk 1.2), waar tevens de mogelijke rol van GABA bij zowel pre- als post-synaptische inhibitie in hersendelen en ruggemerg is besproken (hoofdstuk 1.3). De bewijzen voor een pre-synaptische lokalisatie van de enzymen die de afbraak van GABA verzorgen zijn samengevat in hoofdstuk 1.4. Een verhoogde GABA-erge transmissie resulteerde in een gedragssyndroom dat sterke overeenkomsten heeft met morfine abstinentie gedrag. Daarom is tevens een uitvoerig overzicht gegeven van een aantal aspecten die samenhangen met de acute en chronische toediening van opiaten (hoofdstuk 1.5-1.11).
De aktiviteit van GABA-transaminase en SSA-dehydrogenase is bepaald in totaal hersen homogenaat. GABA-transaminase werd gemeten met behulp van een gekoppelde enzymatische methode waarbij gebruik is gemaakt van het endogeen aanwezige SSA-dehydrogenase om het door GABA-transaminase gevormde SSA verder om te zetten in succinaat. Het gelijktijdig gevormde NADH is gebruikt als een maat voor de aktiviteit van GABA-transaminase. Deze methode kon worden toegepast omdat het dehydrogenase tweemaal zo aktief was als het GABA-transaminase en geen SSA ophoping van betekenis kon worden aangetoond. GABA-transaminase werd geremd door hoge ionsterkte, terwijl NaCl de K_m verlaagde en de V_{max} verhoogde voor SSA-dehydrogenase. Toename van de weefsel concentratie resulteerde eveneens in een verlaging van de K_m terwijl de V_{max} onveranderd blijft. SSA-dehydrogenase kan kennelijk bestaan in twee verschillende aggregatie toestanden: een toestand met een hoge en een toestand met een lage affiniteit voor SSA. De vorm met hoge affiniteit wordt verondersteld akkumulatie van SSA gedurende de GABA-transaminase bepaling te voorkomen. Gekonkludeerd is dat de voorgestelde GABA-transaminase bepaling kan worden toegepast mits zekere voorzorgen worden genomen.
Bakterieel SSA-dehydrogenase is gebruikt als model voor het hersen enzyme, waarbij werd gevonden dat dit enzyme wordt geaktiveerd in de aanwezigheid van monovalente en bivalente kationen. Monovalente kationen als Li^+, Na^+, K^+, Rb^+, Cs^+ of NH_4^+ aktiveerden het enzyme in overeenstemming met hun ion-

straal bij 25 mM, evenals bivalente kationen als Mg^{2+}, Ca^{2+}, Sr^{2+} or Ba^{2+} bij 2.5 mM. De aktivering door monovalente kationen betrof alleen een toename van de V_{max}. Een aktivering of remming kon worden verkregen met Ca^{2+} door het substraat respektievelijk na of voor het kation toe te dienen. Twee vormen van SSA-dehydrogenase met hoge en lage affiniteit zijn in dit preparaat aangetoond. Er is dan ook gekonkludeerd dat het bakteriële enzyme als model kan dienen voor het meer gekompliceerde rattehersen enzyme.
De strukturele voorwaarden voor een remming van SSA-dehydrogenase blijken te verschillen van die voor een remming van het glutamaat decarboxylase (hoofd stuk 2.6). Hieruit is gekonkludeerd dat een selektieve remming van de afbraak van GABA *in vivo* kan worden verkregen door een remming van het SSA-dehydrogenase. Tevens is een model voorgesteld, ontwikkeld op basis van *in vitro* experimenten, dat de selektieve akkumulatie van GABA in het neuronal compartiment zou kunnen verklaren. Dit model is gebaseerd op een selektieve affiniteit van SSA voor GABA-transaminase of SSA-dehydrogenase, wat zou kunnen resulteren in GABA-akkumulatie in het enc en GABA-afbraak in het andere kompartiment (hoofdstuk 2.7).
Toediening van DPA, een selektieve remmer van het SSA-dehydrogenase, aan naieve ratten resulteerde in een quasi-morphine abstinentie gedrag en een toename van de motorische aktiviteit gedurende 15 minuten. Dit gedrag kon worden onderdrukt met een subconvulsieve dosering van 2 mg/kg bicuculline of door voorbehandeling met 5 mg/kg picrotoxine, beide GABA antagonisten, terwijl de selektieve glycine antagonist strychnine geen effekt had. Hieruit blijkt dat het syndroom wordt veroorzaakt door een toename van GABA bij zijn receptor, waaruit kan worden gekonkludeerd dat remming van SSA-dehydrogenase *in vivo* resulteert in GABA afgifte. Een vergelijkbaar syndroom werd niet waargenomen na toediening van de GABA-transaminase remmer AOAA, waaruit kan worden gekonkludeerd dat het effekt van DPA *in vivo* niet door GABA-transaminase remming wordt veroorzaakt. Integendeel, AOAA voorbehandeling onderdrukte het door DPA opgewekte syndroom zodat tenminste twee GABA-systemen in de hersenen moeten bestaan die tegengestelde effekten hebben op het gedrag van de rat. Het syndroom werd tevens tegengegaan door dosering van de GABA synthese remmer 3-MPA, die in onbehandelde ratten convulsies gaven. Het ontstaan van convulsies kon volkomen worden voorkomen door voorbehandeling met DPA, waaruit mag blijken dat de toename van GABA na DPA-behandeling in staat is om de afname van GABA tengevolge van de remming van de GABA-synthese door 3-MPA te kompenseren. Op grond van deze resultaten is een model voorgesteld waarin de GABA-erge zenuwuiteinden worden gekontroleerd door inhibitoire, pre-synaptisch gelokaliseerde GABA-erge auto-receptoren. Deze experimenten met DPA tonen aan dat SSA-dehydrogenase bijdraagt tot de regulering van tenminste één GABA kompartiment, waarmee de werk-hypothese dat SSA-dehydrogenase snelheids bepalende zou kunnen zijn bij de afbraak van

GABA *in vivo* bevestigd is.
De experimenten met morfine en naloxone tonen de betekenis aan van deze proeven voor morfine abstinentie. Toediening van de lage, sub-analgetische dosering van 1 mg/kg morfine resulteerde in een abrupte beëindiging van het door DPA geïnduceerde abstinentie gedrag, terwijl dit weer kan worden opgewekt door toediening van naloxone. Gelijktijdig toedienen van morfine en naloxone had geen effekt. Uit doses-effekt studies is een ED_{50} waarde van 0.5 mg/kg morfine gevonden, waarbij kan worden opgemerkt dat het tijdsverloop van het eigen effekt van morfine van dat van morfine op het door DPA geïnduceerde abstinentie gedrag verschilt. Deze proeven tonen aan dat opiate receptoren betrokken zijn bij de regulatie van het door DPA opgewekte abstinentie gedrag, waarbij tevens kan worden opgemerkt dat de morfine abstinentie geassocieerd is met of veroorzaakt wordt door een verhoogde afgifte van GABA. De waarneming dat een signifikant deel van de ratten 15 min na toediening van DPA kataleptisch waren suggereert dat andere neurotransmitter systemen als het dopaminerge systeem beïnvloed worden door het GABA systeem. De relatie van het GABA-systeem met andere neurotransmitter systemen kan dan ook worden onderzocht door gebruik te maken van het in dit proefschrift beschreven model voor een verhoogde GABA-erge aktiviteit.

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PUBLIKATIES EN VOORDRACHTEN

Een tweetal hoofdstukken van dit proefschrift zijn inmiddels gepubliceerd:

Assay and properties of 4-aminobutyric-2-oxoglutaric acid transaminase and succinic semialdehyde dehydrogenase in rat brain tissue, Th. de Boer and J. Bruinvels, J. Neurochem. 28 (1977) 471-478 (hoofdstuk 2.3).

Suppression of GABA-induced abstinence behaviour in naive rats by morphine and bicuculline. Th. de Boer, H.J. Metselaar and J. Bruinvels, Life Sciences 20 (1977) 933-942 (hoofdstuk 3.3).

Tevens zijn voordrachten gehouden over een aantal hoofdstukken van dit proefschrift, waarvan abstracts zijn gepubliceerd:

Some properties of glutamic acid decarboxylase, GABA-transaminase and succinyl semialdehyde dehydrogenase in rat brain. Th. de Boer, J. Bruinvels and I.L. Bonta, Samenvattingen, symposia en vrije voordrachten van de 16e Federatieve Vergadering, p. 161 (1975) (hoofdstukken 2.2 en 2.3).

Some properties of GAD, GABA-T and SSA-DH in rat brain tissue and the effect of certain drugs. Th. de Boer, J. Bruinvels and I.L. Bonta, Abstracts of the fifth international meeting of the International Society for Neurochemistry, p. 549 (1975) (hoofdstukken 2.2, 2.3 en 2.6).

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De resultaten van de hoofdstukken 2.4 en 2.5 zullen op de "6th international meeting of the International Society for Neurochemistry" in Kopenhagen in de vorm van een poster worden gepubliceerd (augustus 1977).

Publikaties zijn in voorbereiding over de nog niet gepubliceerde gedeelten van dit proefschrift.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 9 december 1947 te Dokkum geboren, doorliep de Openbare Lagere School te Bolsward (1954-1960) en de Rijks-Hogere Burger School (B-richting) te Sneek (1960-1965). Van september 1965 tot januari 1972 werd Scheikunde gestudeerd aan de Rijksuniversiteit te Groningen, waar het kandidaats examen (deel I) in oktober 1967 werd verkregen. Vervolgens werd in januari 1972 het doktoraal examen afgelegd met als hoofdvak Biochemie (Prof. Dr. M. Gruber en Dr. J.M.W. Bouma) en met als bijvak Biologische Psychiatrie (Prof. Dr. H.M. van Praag en Dr. J. Korf). Na een jaar waarin de militaire dienstplicht werd vervuld, is tussen februari 1973 en september 1977 op de Afdeling Farmacologie van de Erasmus Universiteit te Rotterdam (Hoofd: Prof. Dr. I.L. Bonta) onder begeleiding van Dr. J. Bruinvels dit proefschrift bewerkt en was schrijver dezes als wetenschappelijk medewerker aan deze afdeling verbonden.