THE OCCURRENCE OF β-CARBOLINES IN MAN AND RAT, PUTATIVE BIOCHEMICAL SUBSTRATES RESPONSIBLE FOR PSYCHOSIS

Het voorkomen van β-carbolines in de mens en in de rat: mogelijke biochemische substraten verantwoordelijk voor psychose

PROEFSCHRIFT

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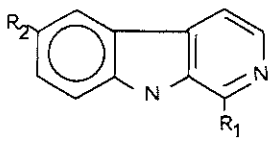
Chapter I

1.1. INTRODUCTION

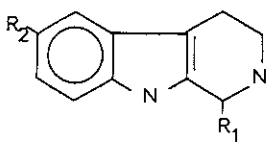
Theories concerning the etiology of psychosis have, over the last century, focussed upon environmental, cultural, sociological, psychological, morphological, biological and biochemical factors which may predispose an individual to the development of a psychosis (Barker, 1980). While there is to some extent supporting evidence for each hypothesis, no single idea has been generally accepted. This has led to the description of psychosis as a heterogenous disease or syndrome with many possible etiologies. During the last fifty years reproducible results from different disciplines have begun to yield clues to some of the possible biochemical processes involved in this group of diseases (Smythies, 1982). There has been a constant refinement of diagnostic procedures and a better clinical understanding of the symptomatology associated with the disease. At present it seems likely that the term psychosis still applies to a group of etiologically separate but symptomologically similar diseases. Therefore, several biochemical theories on the etiology of psychosis coexist, each of which is more or less supported by experimental evidence, despite these theories being somewhat contradictory at first glance (Barker, 1980).

In paragraph 1.2 of this chapter, some hypotheses of psychosis related to the one-carbon cycle, are described. In paragraph 1.2.1. and 1.2.2. the transmethylation and one-carbon cycle hypothesis are respectively described, as originally put forward by Osmond and Smythies (1952). These hypotheses suggest a faulty methylation of biogenic amines in the one-carbon cycle, or an altered activity of some of the enzymes in this cycle, as a possible cause of schizophrenia. In paragraph 1.2.3. possible pathways in mammals for the formation of beta-carbolines, potentially hallucinogenic compounds, are described. In the last part of paragraph 1.2. some metabolic aberrations in serine metabolism are described in a group of patients suffering from an acute psychosis of the manic psychedelic type (Pepplinkhuizen, 1983), giving support to the hypothesis that hallucinogenic compounds could be formed endogenously during acute psychosis. It was hypothesized that a disturbance in the serine- and glycine metabolism could give rise to excess formation of formaldehyde, and thus possibly to the formation of tricyclic compounds with a beta-carboline structure (see Fig. 1), some of which have known hallucinogenic properties. The occurrence of beta-carbolines in man and rat is described in paragraph 1.3. The last part of this chapter gives a description of the methods which have been used to investigate whether the metabolic alterations in the serine-

and glycine metabolism can evoke behavioural disturbances in rats, and whether these compounds are actually being formed in this animal model, as well as a description of the methods used to measure beta-carbolines in man during psychosis.



A



B

structure	$-R_1$	$-R_2$	name
A	-H	-H	norharman
A	$-CH_3$	-H	harman
B	-H	-H	THBC
B	-H	-OH	6-hydroxy-THBC
B	-H	$-OCH_3$	6-methoxy-THBC

Fig. 1. A: beta-carboline (BC) and **B:** 1,2,3,4-tetrahydro-beta-carboline (THBC) parent structure.

1.2. HYPOTHESES OF PSYCHOSIS RELATED TO THE ONE-CARBON CYCLE

1.2.1. The transmethylation hypothesis.

All transmethylation reactions of biogenic amines in the mammalian body receive methyl groups via S-adenosylmethionine, (SAM), generated in the one-carbon cycle, as depicted in Fig. 2. SAM is synthesized from adenosine and methionine by the enzyme

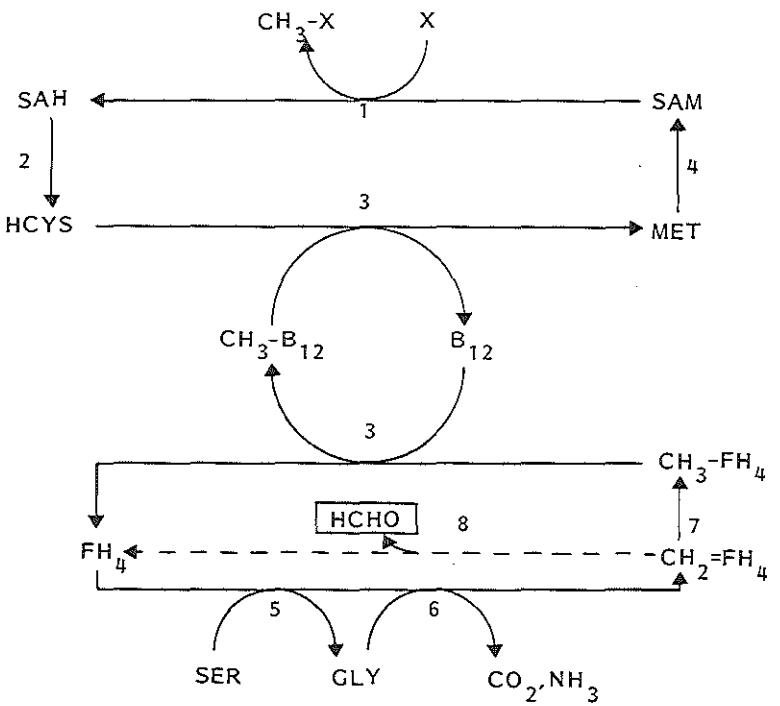


Fig. 2. The one-carbon cycle.

SAM	= S-adenosylmethionine	1.	O-methyltransferases
SAH	= S-adenosylhomocysteine	2.	Adenosylhomocysteinase
HCYS	= homocysteine	3.	N-methyl tetrahydrofolate: homocysteine methyltransferase
MET	= methionine	4.	methionine adenosyltransferase
B_{12}	= vitamin B_{12}	5.	serine hydroxymethyltransferase
CH_3-FH_4	= methyltetrahydrofolate	6.	glycine cleavage system
CH_2=FH_4	= methylenetetrahydrofolate	7.	methylene tetrahydrofolate reductase
FH_4	= tetrahydrofolate	8.	non-enzymatic
HCHO	= formaldehyde		
SER	= serine		
GLY	= glycine		

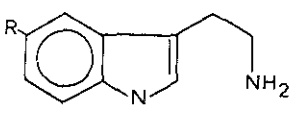
methionine adenosyl transferase (MAT). The most important one-carbon donor is serine, by conversion to glycine, forming methylenetetrahydrofolic acid (CH_2=FH_4). This compound is dehydrogenated to methyltetrahydrofolic acid, which can donate it's methylgroup to S-adenosylhomocysteine (SAH) in a vitamin B_{12}-dependent reaction.

The original transmethylation hypothesis of schizophrenia (Osmond and Smythies, 1952) was based on the fact that mescaline, a psychotogenic compound, showed a structural resemblance to O-methylated cathecholamines. They suggested that a disturbance of brain catecholamine methylation could lead to the formation of psychotogenic substances by an overactive transmethylation system. In 1961, Pollin et al. discovered that oral administration of methionine in schizophrenic patients could evoke psychopathological effects, and/or a superimposed toxic psychosis in schizophrenic patients. These findings were confirmed by several other investigators, as reviewed by Cohen et al. (1974), who concluded that the administration of methionine has profound effects on mood and perception in 40 % of chronic schizophrenics. These findings supported the transmethylation hypothesis to some extent, because methionine is the main precursor of SAM, an important methyldonor (Fig. 2). The authors concluded that an aberrant transmethylation of biogenic amine precursors could play some role in the pathogenesis of schizophrenia (Cohen et al., 1974). Many of the methionine-loaded schizophrenics did not manifest the symptoms described above within several hours, as would be expected after the endogenous formation of "psychotogenic" compounds, but only after several days of chronic administration of this amino acid. However, the effects exerted by methionine may point to some basic fault in the transmethylation mechanisms in the one-carbon cycle (Smythies, 1982).

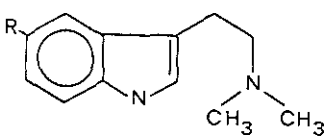
The hypothesis of methylation disorder was given support by Brune and Himwich (1962), who obtained comparable results after substitution of betaine for methionine in the loading experiment. Betaine is also a methyldonor, but of a chemical family quite different from methionine.

As reviewed by Smythies (1984a), the transmethylation hypothesis was extended to include the production of abnormal hallucinogenic metabolites of serotonin or tryptamine, viz. dimethyltryptamine (DMT) and O-methyl bufotenine (OMB, Fig. 3) of which the latter is known to be hallucinogenic in man (Gillin, 1978). Several aberrations in one-carbon methylation mechanisms have been found since (Smythies, 1984a). It has been shown that SAM is a source of methyl groups for the enzymatic methylation of

hydroxyl- and nitrogen groups of biogenic amines in lung tissue (Axelrod, 1961). Also, methionine and SAM were supposed to be the indirect and direct methyl donor in N,N-dimethylations of serotonin and tryptamine, forming bufotenine and dimethyltryptamine, respectively (DMT, Axelrod, 1961; Mandell and Morgan, 1971). It has been reported that an enzyme, found in human brain could synthesize DMT from tryptamine via a transmethylation reaction (Mandell and Morgan, 1971). Another line of possible evidence was provided by Poitou (1974), who found that cathecholamine-O-methyl transferase was elevated in red blood cell ghosts in schizophrenics, but these results could not be replicated by others (Gillin, 1978).

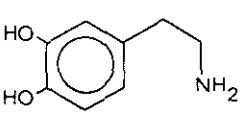


parent amine

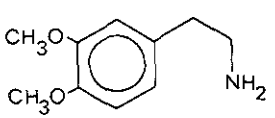


schizotoxin

-R	parent amine	schizotoxin
-H	tryptamine	dimethyltryptamine (DMT)
$-OCH_3$	5-MeO-tryptamine	5-MeO-DMT (O-methylbufotenin)
-OH	serotonin	bufotenin



dopamine



dimethoxyphenylethylamine (DMPEA)

Fig. 3. Putative hallucinogenic compounds and their precursors formed by methylation processes.

Until now, the search for psychotogenic compounds which could be endogenously formed by an increased one-carbon cycle activity has not been very succesful. Although DMT and OMB have been identified in human CSF and rat brain by gas-chromatography/mass-spectrometry (GC/MS, Smythies et al., 1979), and in urine using a GC technique (Oon et al., 1977), no differences between schizophrenics and normal controls were found. Moreover, much

higher amounts of DMT were excreted into urine in cases of liver disease without psychosis (Checkley et al., 1979). In addition, rat brain DMT and OMB are known to be increased significantly by the induction of stress, and this increase could be prevented by adrenalectomy (Beaton and Christian, 1978; Harrison, 1982). This suggests a physiological role of DMT and OMB related to stress rather than to psychosis (Smythies, 1983) . Other studies have shown that methionine administration did not increase urine levels of methylated metabolites of cathecholamines in man (Antun et al., 1971), nor did it increase the production of DMT in rat brain after methionine or SAM administration. It actually decreased the production of DMT by rabbit lung (Stramentinoli and Baldessarini, 1978). This was in accordance with the findings of Baldessarini (1978), which indicated that excess methionine in fact inhibits transmethylation, which was explained by the fact that SAH, one of the products of the transmethylation reaction (Fig. 2), is a potent product inhibitor. Therefore, it can be concluded that there is no existing evidence which shows that putative endogenous hallucinogens can be formed as a result of aberrant transmethylation reactions (Smythies, 1983).

1.2.2. The one-carbon cycle hypothesis.

In 1963 the transmethylation hypothesis was modified into the one-carbon cycle hypothesis, which suggested a fault in the methylation process itself, rather than in the formation of psychotogenic products (Smythies, 1963). Carl et al. (1978) showed that the V_{max} of methionine adenosyltranferase (MAT) and that of serine hydroxymethyltransferase (SHMT, Fig. 2) are significantly reduced in a group of schizophrenics, whereas two other enzymes of the one-carbon cycle did not differ in activity from controls. However, patients low in MAT activity were not the same as those low in SHMT activity, which points to different schizophrenic subgroups. Israelstam et al. (1970) determined the overall rate of transmethylation by measuring the evolution of $^{14}CO_2$ after administration of ^{14}C-methionine. It was found that this process was slower in schizophrenic patients when compared to controls, whereas it was much faster in depressives, which were on tricyclic anti-depressant therapy. Ismail et al. (1978) confirmed these findings in schizophrenics using in vitro experiments, showing a decreased oxidation of the methyl-carbon of methionine in the leukocytes of schizophrenic patients. These results suggested that schizophrenia and depression can be associated in some cases with an underactive methylation system, rather than with the formation of abnormally methylated products as a result of increased activity of the one-carbon cycle (Ismail

et al., 1978).

More evidence that methylation processes may be altered in some affective disorders was collected by Fazio et al. (1976), who used S-adenosylmethionine (SAM) to investigate the transmethylation hypothesis. They noted that SAM had antidepressant properties. Further studies indicated that both unipolar and bipolar depressives also had a significantly lowered MAT function, whereas the V_{max} for MAT was elevated in four of six manic patients (Tolbert et al., 1983). These changes were not influenced by neuroleptic medication, age or sex variables. They have suggested that those depressed patients who respond to SAM are those with a low V_{max} for MAT. In addition, SAM not only has antidepressant properties, but can also cause a switch into mania in bipolar patients. It has been suggested that some depressives react to SAM because of defective production of their own SAM due to low MAT activity, and that mania may be associated with high MAT activity (Tolbert et al., 1984; Smythies, 1984b). Furthermore, it was suggested that the exacerbative effect of methionine in schizophrenic patients may be a manic reaction superimposed on the schizophrenic process (Tolbert et al., 1984).

In conclusion, the former idea that an aberrant methylation of biogenic amines through an increased activity of one-carbon cycle enzymes is involved in the etiology of schizophrenia is not supported by biochemical findings. However, decreased activity of MAT and SHMT was found in schizophrenic subgroups, as well as an antidepressant effect of SAM in depressed patients, which indicates that alterations in the kinetics of the transmethylation system in the one-carbon cycle may play an important role in schizophrenia and affective illness.

1.2.3. How can hallucinogenic compounds be formed?

As described above, since Osmond, Harley-Mason and Smythies have put forward their transmethylation hypothesis, no evidence has become available that hallucinogenic compounds are formed endogenously during psychoses (Smythies, 1984b). Although some compounds like DMT, OMB and also dimethoxyphenylethylamine (DMPEA, the 'pink spot') have been detected in the urine of schizophrenics, these results were irreproducible by others or the amounts found were not different from normal values (Gillin et al., 1978).

In order to find a biochemical rationale for the exacerbative effect of methionine in schizophrenic patients, several

enzymes were described which could be responsible for the formation of hallucinogenic compounds during psychosis. Axelrod (1961) described an enzyme isolated from rabbit lung which could convert tryptamine to DMT using SAM as a methyldonor. Mandell and Morgan (1971) reported the presence of an indoleethylamine N-methyltransferase in human brain, which also used SAM as the methyldonor and Laduron (1972) described an N-methyltransferase with methyl tetrahydrofolic acid (CH_3-FH_4) as a methyl donor. These mechanisms could explain the methionine effect in two ways: (1) Methionine can be converted to SAM, which can act directly as a methyl donor in N- or O-methylation of catecholamines or indoleamines, and (2) methionine can inhibit the methylation of homocysteine, which may result in accumulation of CH_3-FH_4 (Fig. 2). The excessive production of these two methyldonors could thus account for the formation of hallucinogenic compounds via aberrant methylation reactions. However, more careful studies of the reaction mechanism revealed that N^5,N^{10}-methylenetetrahydrofolic acid (CH_2=FH_4) instead of CH_3-FH_4 is the ultimate methyl donor in this pathway (Meller et al., 1975). This compound can be formed by the reverse reaction of N^5,N^{10}-methylenetetrahydrofolate reductase, and can non-enzymatically decompose in tetrahydrofolic acid and formaldehyde. The latter compound can condense in the so-called Pictet-Spengler reaction with indoleethylamines or cathecholamines forming tetrahydro-beta-carbolines (THBC) or tetrahydroisoquinolines (THIQ), respectively (Fig. 4, Leysen and Laduron, 1974; Pearson and Turner, 1975; Taylor and Hanna, 1975).

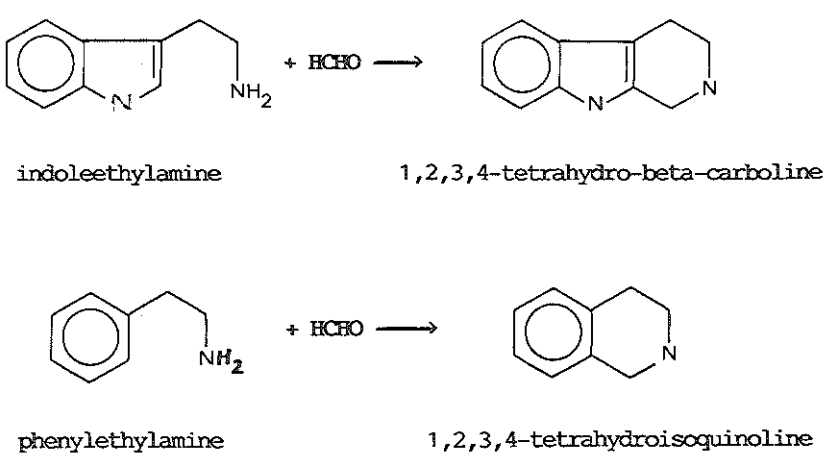


Fig. 4. The Pictet-Spengler reaction.

Indeed, careful analysis of the reaction products using CH_3-FH_4 as the methyldonor showed that these were not the expected N- or O-methylated indoles, but tetrahydro-beta-carbolines (Mandel et al., 1974). These results are in agreement with the suggestion made by Bruinvels (1975), that CH_2=FH_4, rather than CH_3-FH_4, is involved in the development of acute psychotic effects in schizophrenic patients.

The Pictet-Spengler reaction between indoleethylamines and some aldehydes, depicted in Fig. 4, has been known for several decades, and it was shown by Hahn and Ludevig (1934) that this reaction occurs easily at physiological conditions (37 °C and neutral pH). More support for the suggestion that CH_2=FH_4 is the methyldonor in aberrant methylating reactions was gained by the findings of Pearson and Turner (1979) who revealed that THBC can be formed by incubating partially purified serine hydroxymethyltransferase (SHMT) with serine, FH_4 and tryptamine. Under the conditions used, the beta-carbon of serine is transferred to FH_4 forming CH_2=FH_4, which yielded THBC as described above. These findings gave support to the early hypothesis of McIsaac (1961), who was the first to postulate that endogenous formation of beta-carbolines could be involved in mental diseases, as well as to the hypothesis that 'hyperformaldehydism' may be involved in the etiology of schizophrenia (Fig. 5; Barker, 1980).

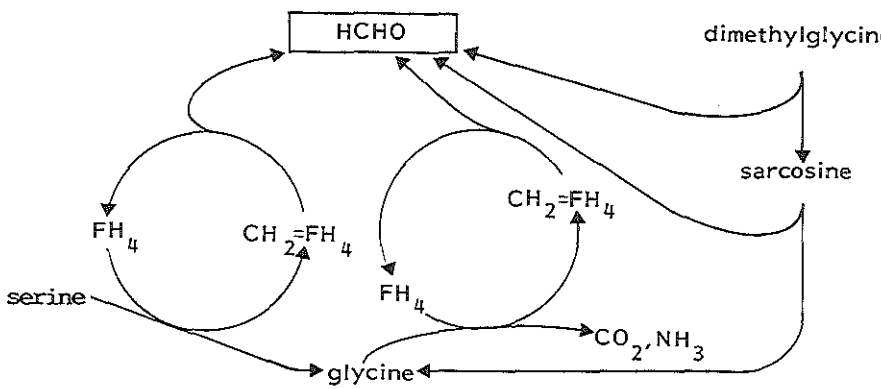


Fig. 5. Formation of formaldehyde in the mammalian body.

1.2.4. The serine hypothesis.

In 1980, Pepplinkhuizen et al. described four patients suffering from schizophrenia-like psychosis which resembled closely

a hallucinogenic drug-induced state. These patients were suspected of having a porphyric disease, which was held responsible for a disturbed serine and glycine metabolism (Fig. 6). Oral loading tests using 2 mmol serine per kg body weight resulted in acute psychedelic or psychotic symptoms within 5 hours, and lasted for 3-6 hours. Decreased excretion of serine into the urine was measured in these patients, suggesting increased consumption of this amino acid. During later experiments, on the basis of hourly measured amino acid plasma levels over a period of 6 h after oral serine- or glycine loading tests, altered serine- and glycine plasma levels were found when compared with controls. These findings, which were in some cases coupled with acute porphyria, may be responsible for an increased formation of CH_2=FH_4 (Bruinvels et al., 1980). In acute porphyria, the biosynthesis of delta-aminolevulinic acid (d-ALA) is strongly enhanced. Since d-ALA is synthesized from glycine and succinyl-CoA (Fig. 6), the demand for glycine will be increased under these circumstances. Consequently, more serine is converted into glycine via serine hydroxymethyltransferase (SHMT) using FH_4 as a cofactor, which will concomitantly increase the production of CH_2=FH_4. This theo-

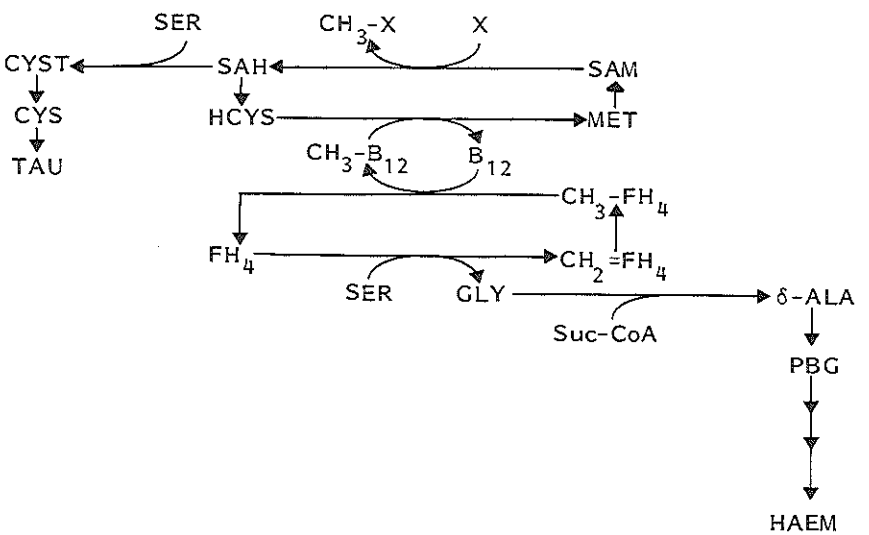


Fig. 6. Metabolic relationships between taurine- and porphyrin pathways and the one-carbon cycle.

CYST = cystathionine
CYS = cysteine
Suc-CoA = succinyl-CoA
d-ALA = delta-aminolevulinic acid
PBG = porphobilinogen
TAU = taurine

ry was supported by the beneficial effects of treatments which are also used in the treatment of acute porphyria, eg. the consumption of carbohydrate-rich diet and administration of glucose, which were found to be superior to treatment with neuroleptics (Pepplinkhuizen, 1983). In one patient, treatment with vitamin B_{12}, which has also benificial effects in porphyric diseases, improved psychotic symptoms as well. In the same patient, both psychotic and porphyric symptoms were precipitated after treatment with barbiturates (Pepplinkhuizen, 1983; Pepplinkhuizen and Wunderink, 1985). Moreover, the psychiatric symptoms which are often seen during acute porphyric attacks resembled the symptoms of episodic acute psychoses (Pepplinkhuizen and Wunderink, 1985; Goldberg and Stinnet, 1983).

Oral administration of the amino acids serine or glycine to patients suffering from this type of psychosis revealed that the conversion of glycine to serine was impaired (Bruinvels and Pepplinkhuizen, 1984). Moreover, taurine plasma levels were increased and serine plasma levels were decreased in fasting, serine-responsive, episodic psychotic patients (Bruinvels and Pepplinkhuizen, 1984). These results were interpreted as the increased taurine formation being the primary cause of the derangement in serine-responsive patients, which in turn would lead to a decrease in serine plasma levels (Fig. 6). It was suggested that limited activity of methyltetrahydrofolate:homocysteine methyltransferase in serine positive patients could result in the accumulation of CH_2=FH_4 after serine loading of these patients (Fig. 6). This in turn would lead to the formation of tetrahydro-beta-carbolines or tetrahydroquinolines via the dissociation of CH_2=FH_4 into formaldehyde and FH_4 (Fig. 5) and the subsequent condensation with indoleamines or catecholamines, respectively (Fig. 4). These substances may be responsible for the psychiatric symptoms seen during acute psychotic attacks, or after serine loading in patients. It remains, however, to be shown whether hallucinogenic beta-carbolines or tetrahydroisoquinolines are indeed being formed during this type of episodic psychosis.

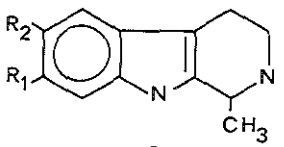
1.3. OCCURRENCE AND EFFECTS OF beta-CARBOLINES IN MAMMALS

Despite the fact that several metabolic aberrations in psychotic and schizophrenic patients have been described , it has, until now, not been shown that hallucinogenic compounds, formed in mammalian species, could be held responsible for the psychiatric symptoms in these patients. General interest in beta-carbolines has been increased since the proposal of McIsaac (1961 a and b) that these compounds could be involved in the development of mental disease. This interest has mainly been prompted by the following findings:

Ethnopharmacology. Beta-carbolines are found in plants which have been used as hallucinogens by tribes living in various parts of South-America, Asia and Africa, who were familiar with the potent psychoactivity of several plants containing beta-carbolines (Schultes, 1982, List and Hoerhammer, 1977; Naranjo, 1979). Also ancient Greeks and Europeans in our centuries have been using seeds of Peganum Harmala in the treatment of eye disease, as analgesicum and aphrodisiac, as a "truth serum", or as an antidepressive. These seeds contain several beta-carbolines, such as harmaline (7-MeO-1-Me-DHBC), harmalol (7-OH-1-Me-DHBC) and harmine (7-Me-1-Me-BC), among others (Fig. 7, Airaksinen and Kari, 1981 I and II). West-American natives have used extracts from Passiflora species (edulis and incarnata) for several indications eg. insomnia, spasms and cramps (Airaksinen and Kari, 1981 II). Narcotic and hallucinogenic agents containing beta-carbolines, which were prepared from the South-American lian banisteriopsis caapi and other plants of the Malpighiaceae, have been used by South-American Indians (Airaksinen and Kari, 1981 II).

Hallucinogenic properties. Some pure beta-carbolines have known hallucinogenic properties. The largest study on the hallucinogenic effects of these compounds in man has been carried out by Naranjo (1967, 1979), who orally administered harmaline (7-MeO-1-Me-DHBC), harmine (7-MeO-1-Me-BC), tetrahydroharmine (7-MeO-1-Me-THBC), 6-methoxyharmalan (6-MeO-1-Me-DHBC) and 6-methoxytetrahydroharman (6-MeO-1-Me-THBC, see Fig. 7) to healthy volunteers. When the subjects had their eyes closed, they described vivid, coloured imagery (closed eye imagery). When eyes were open, they described the superposition of images on walls or ceilings, or the viewing of imaginary scenes simultaneously with an undistorted perception of surrounding objects. Harmaline was found to be even more hallucinogenic than mescaline, which is the most visually acting drug in it's chemical group. Doses needed

for these effects ranged from 4-15 mg/kg when administered orally.



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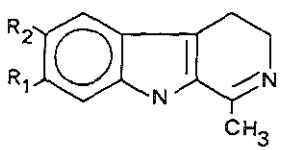

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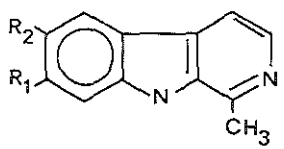

C

compound	structure	$-R_1$	$-R_2$
harmaline	B	$-OCH_3$	-H
harmine	C	$-OCH_3$	-H
tetrahydroharmine	A	$-OCH_3$	-H
6-methoxyharmalan	B	-H	$-OCH_3$
6-methoxytetrahydroharman	A	-H	$-OCH_3$

Fig. 7. beta-Carbolines administered to humans.

Physiological occurrence. Tetrahydro-beta-carboline- and beta-carboline derivatives have been detected in human and rat tissues and body fluids, as can be seen in Table 1 and 2. Beta-carbolines and tetrahydro-beta-carbolines in man were mainly found in plasma and platelets. Considerable differences in concentration of some beta-carbolines were reported, even by the same group of investigators, which could be due to artifactual formation of beta-carbolines during the work-up procedures. Some doubt still exists as to their physiological occurrence, because these compounds are very easily formed from formaldehyde and indoleamines or catecholamines, as discussed by Bosin et al. (1983) and Bloom and Barchas (1982). It was shown by Bosin et al. (1983) that 6-OH-THBC can be formed during sample preparation due to the presence of formaldehyde in organic solvents. To the authors' knowledge, there is only one article reporting the measurement of beta-carboline in acute schizophrenic patients

Compound	locus	conc	dimension	reference
THBC	platelets	5.8	pm/10^8 plat.	Honecker 1978b
	,,	10.6	,,	Kari 1979
	,,	0.5	,,	Honecker 1980
	,,	20	,,	Barker 1982
	plasma	31.4 ± 5.1	pm/ml	Kari 1979
	urine	232 - 6163	pm/sample	Honecker 1978a
6-OH-THBC	platelets	2.7 ± 0.3	pm/10^8 plat.	Rommelsp. 1982
	,,	13.2	pm/sample	Bosin 1983
6-MeO-THBC	platelets	7.5	pm/10^8 plat.	Barker 1982
	plasma	6.2 ± 1.7	pm/ml	Rimon 1984
	retina	9	pm/gr	Leino 1984
6-OH-1-CH_3-THBC	urine	5.1 ± 1.3	pm/um creat.	Beck 1982
1-Me-BC	platelets	detected	-	Bidder 1979

Table 1. beta-Carbolines (concentrations ± standard error of the mean (SEM)) in human tissues and body fluids (pm=picomole; um=micromole; plat.= platelets; creat.= creatinine).

Compound	locus	conc	dimension	reference
THBC	brain	11.8 ± 2.9	pm/gr wet wt.	Barker 1979
	,,	101.7 ± 28.5	,,	,, 1981
	,,	0.23	,,	Johnson 1984
	forebrain	275 ± 78	,,	Honecker 1978a
6-OH-THBC	platelets	275 ± 129	pm/10^8 plat.	Rommelsp. 1979
	,,	3.2 - 6.9	,,	,, 1982
6-MeO-THBC	brain	176 ± 82	pm/gr wet wt	Barker 1981
	adrenal	5513 ± 148	,,	,, ,,
	arc. nucleus	detected	-	Shoemaker 1978
6-OH-1-CH_3-THBC	plasma	0.52 ± 0.15	pm/ml plasma	Beck 1983
	urine	3.6 ± 1.0	pm/24 hr	,, ,,
	faeces	0.9 ± 0.1	,,	,, ,,
	liver	11.1 ± 3.6	pm/gr liver	,, ,,
1-Me-BC	arc. nucl.	1300	pm/gr arc. nucl.	Shoemaker 1980

Table 2. beta-Carbolines (concentrations ± SEM) in rat tissues and body fluids (pm=picomole; plat.= platelets).

(Rimon et al., 1984). This group has measured 'pinoline' or 6-methoxy-1,2,3,4-tetrahydro-beta-carboline levels in serum and cerebrospinal fluid of schizophrenic patients. A detectable concentration of 6-MeO-THBC was always present, but no differences were found between the schizophrenic and the control groups. Besides, these values were not related to different subtypes of schizophrenia, or to the intensity of the psychopathological symptoms.

Monoamine oxidase inhibition. Monoamine oxidase is the principal enzyme concerned with the inactivation of serotonin (5-HT) and other neurotransmitters. Monoamine oxidase inhibitors have been used as antidepressants for several decades, and are thought to function via increases in availability of monoamine neurotransmitters at synapses in the central nervous system (Bowman and Rand, 1980). Low platelet monoamine oxidase (MAO) activity has been considered as a biological marker in schizophrenia, as reviewed by Siever and Coursey (1985). It has been found that there is a mild decrease in platelet MAO-activity among chronic schizophrenic groups, but not in acute schizophrenia. However, long-term neuroleptic medication and cigarette smoking have appeared to be serious artifacts in this relationship. Moreover, MAO-activity did not vary with changes in clinical symptoms. Therefore, some doubt still exists on the value of MAO-activity as a biological marker (Siever and Coursey, 1985). Nevertheless, the observation that beta-carbolines are potent and selective MAO-inhibitors, is quite intriguing in this respect. The inhibitory effect of beta-carbolines on MAO, mainly of the A-type, has been shown to be reversible and usually competitive (Airaksinen and Kari, 1981 II). In general, the unsaturated beta-carbolines are more potent MAO-inhibitors than the corresponding tetrahydro-beta-carbolines, as shown in liver and brain tissue (Airaksinen and Kari, 1981 II). IC_{50}-values of the most potent MAO-inhibiting beta-carboline (harmaline) is about 10^{-8} M, and about 10^{-5} M for the most potent tetrahydro-beta-carboline (Buckholtz and Boggan, 1977). Moreover, it has been shown in rats that the concentration of brain 5-hydroxyindoleacetic acid (5-HIAA) decreased after the administration of beta-carbolines, which suggests that beta-carbolines in vivo may act by inhibition of MAO (Airaksininen and Kari, 1981 II).

5-HT reuptake inhibition. A dysfunction in central serotonin (5-HT) metabolism in schizophrenia has been repeatedly suggested (DeLisi et al., 1981). Data have been obtained by measuring the concentrations of 5-HT and it's metabolites in urine and blood (Stahl et al., 1982), in post-mortem brain, in cerebrospinal fluid, and after administration to schizophrenic patients of 5-HT

precursors and/or drugs affecting central 5-HT turnover (De Lisi et al., 1981). It was concluded from these experiments that increased whole blood 5-HT concentrations in chronic schizophrenic patients could be used as a biological marker for schizophrenia. Therefore, it is interesting that beta-carbolines inhibit the 5-HT reuptake, 6-OH-THBC being the most active in synaptosomes and 6-MeO-THBC in platelets, but only the latter slightly exceeding the potency of 5-HT. 1-Methylated THBCs and all DHBCs and BCs studied were less potent (Airaksinen and Mikkonen, 1980; Komulainen et al., 1980; Rommelspacher et al., 1978). Therefore, reuptake inhibition may be the mechanism by which beta-carbolines increase 5-HT concentrations in the synaptic cleft. The high selectivity of some beta-carbolines in increasing 5-HT concentrations in rat brain may result from the combination of selective MAO-A and 5-HT reuptake inhibition, respectively, which allows them to inhibit brain MAO effectively only in 5-HT neurons (Airaksinen and Kari, 1981 II). It was proposed by Langer et al. (1984) that 6-MeO-THBC may subserve the role of a hormone that modulates the neuronal uptake of 5-HT, and which is probably synthesized, stored and released by the pineal gland to fulfill this physiological function. They speculated that 6-MeO-THBC could play an important role in the pathophysiology of depression.

Benzodiazepine receptor binding. The discovery of high affinity, saturable and stereospecific receptors for benzodiazepines in the mammalian central nervous system has led to an intensive search for endogenous factors that physiologically regulate this receptor (Cain et al., 1982). Although Braestrup et al. (1980) originally proposed beta-carboline-3-carboxylic acid ethyl ester (BCEE) as the endogenous factor, subsequent studies have shown that this compound is probably formed during the isolation and extraction procedure (Nielsen et al., 1982). However, it has now definitely been established that beta-carbolines have high affinity for the benzodiazepine receptor (Cain et al., 1982). It has been demonstrated that BCEE antagonizes the anticonvulsant action of diazepam. Cain et al. (1982) have reported on the interactions of a series of beta-carbolines and tetrahydro-beta-carbolines with benzodiazepines in vitro. Beta-carboline-3-carboxylic acid propyl ester was most potent in displacing [^{3}H]diazepam (K_I=1 nM). Removal of the carbonyl substituent for the 3-position resulted in a decrease in affinity (K_I= 1.6 microM for norharman). The introduction of a methyl group in the 1-position further decreased affinity for the benzodiazepine receptor (K_I= 12.4 microM for harman). The corresponding saturated tetrahydro-beta-carbolines are, in general, a hundred times less potent in benzodiazepine receptor binding than their non-saturated counterparts

(Cain et al., 1982). The observation that B-CEE elicits tonic and clonic convulsions in rodents, demonstrated that beta-carbolines not only antagonize the principal pharmacological actions of benzodiazepines, but also produce intrinsic actions which are more or less opposite to the benzodiazepines. These compounds have been termed "inverse agonists" or "active antagonists" (Skolnick et al., 1984b). Administration of B-CEE to chair-adapted, male rhesus monkeys elicited anxious behaviour, which was suggested to be a reliable and reproducible model of human anxiety (Skolnick et al., 1984a). As shown by studies of Morin (1984), norharman produces kindled seizures when given daily at a subconvulsive dose of 20 mg/kg intraperitoneally. These effects could be blocked by diazepam and other ligands of the bezodiazepine receptor. These results indicate that in vivo effects of beta-carbolines are probably mediated by the benzodiazepine receptor (Morin, 1984).

Summarizing, there is evidence to suggest the presence of saturated and non-saturated beta-carbolines in mammalian tissues. However, this research may include artifactual formation of beta-carbolines during work-up procedures. Besides, these compounds are pharmacologically very active as MAO-A inhibitors, 5-HT reuptake inhibitors and inverse agonists or antagonists of the benzodiazepine receptor, suggesting a role as neuromodulator for these compounds. However, despite their interesting pathopharmacological profile, there is as yet no evidence that these compounds are formed in substantial amounts during mental diseases.

1.4. METHODS OF INVESTIGATION

Animal model for psychosis. As hypothesized above, an increased conversion of serine into glycine, resulting in increased production of $CH_2=FH_4$, could give rise to the production of beta-carbolines or tetrahydroisoquinolines via the reaction of formaldehyde with biogenic amines. Based on this hypothesis it was tried to develop an animal model in which the putative formation of these compounds could be measured and correlated with behavioural disturbances. Induction of porphyria by chemical methods was used to increase the conversion of serine into glycine by induction of delta-aminolevulinic acid synthetase, the first enzyme in the heme pathway. It was assumed that cataleptic and/or catatonic behaviour could be used as an indicator for psychotic behaviour (Bloom et al., 1972; Maxwell, 1968; Segal et al., 1977 and Chapter II). As described in chapter II, behavioural disturbances could indeed be measured in porphyric rats after serine or glycine treatment.

Measurement of beta-carbolines in man and rat. As can be seen from Tables 1 and 2, beta-carbolines have been measured in human and animal tissues in variable quantities. However, these variations are probably due to the artifactual formation of formaldehyde during sampling, processing and quantitation of these tissue samples. To cope with these problems, a new method was developed in order to avoid the usage of chemicals or procedures which could introduce formaldehyde into the samples. A description of this method is given in Chapter III.

Formation of beta-carbolines in porphyric rats. In order to test the hypothesis that beta-carboline compounds are formed in rats during increased serine to glycine conversion, some of these compounds were measured in platelet rich plasma, using the method described in Chapter III. As described in Chapter IV, a non-saturated beta-carboline was detected in platelet rich plasma after treatments described in Chapter II.

Formation of beta-carbolines during psychosis. Despite the fact that many metabolic aberrations have been described in psychotic and schizophrenic patients, it has, until now, not been shown whether hallucinogenic compounds are endogenously formed, which could account for the psychiatric symptoms of these patients. In order to test the hypothesis that this type of compound can be formed during acute episodic psychosis of the manic psychedelic type, beta-carbolines and tetrahydro-beta-carbolines were measured in blood samples of patients, suffering from acute

psychosis on the moment of blood withdrawal.

CHAPTER II

SERINE AND GLYCINE-INDUCED CATALEPSY IN PORPHYRIC RATS; AN ANIMAL MODEL FOR PSYCHOSIS?

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by

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Summary

It was investigated whether an increased demand for glycine, as postulated to occur in patients who have suffered from episodic psychoses accompanied by multiple perceptual distortions, could evoke psychotic reactions. Catalepsy was used as a measure for psychosis, and was observed after injection of serine or glycine in porphyric rats. Catalepsy was shown to occur after serine as well as glycine administration in 2-allyl-2-isopropylacetamide (AIA) pretreated rats, while in lead + phenobarbital pretreated rats only glycine was effective. Administration of AIA to rats resulted in a strongly enhanced excretion of porphobilinogen (PBG) in urine, while lead + phenobarbital pretreated rats showed increased excretion of d-aminolevulinic acid (d-ALA). The lead + phenobarbital pretreated animals showed elevated serine plasma levels and lowered glycine plasma levels 18 hours after injection, while no significant differences in plasma levels of these amino acids were found 24 hours after AIA administration. In AIA or saline pretreated animals, but not in those pretreated with lead + phenobarbital, glycine formation from serine was elevated. It is concluded that the present animal model can be used to investigate episodic psychoses.

Chapter II

INTRODUCTION

It has been generally accepted that the effects of hallucinogens in man bear a large resemblance to the symptoms of an acute psychosis. During the past two decades many suggestions have been made about the possible endogenous formation of hallucinogenic or even "schizophrenic" compounds. Osmond and Smythies (1952) were the first to postulate that a false methylation of catecholamines may form a biological basis for schizophrenia.

It has been suggested previously that an increased production of methylenetetrahydrofolic acid (CH_2=FH_4) and not methyltetrahydrofolic acid (CH_3-FH_4) could be involved in the development of schizophrenia (Bruinvels, 1975). Under physiological conditions this compound can be non-enzymatically decomposed into FH_4 and formaldehyde (HCHO, Donaldson and Keresztesy, 1961) of which the latter can react spontaneously via the Pictet-Spengler reaction with indolamines or catecholamines forming THBC's or THIQ's respectively (Airaksinen and Kari, 1981 I; Deitrich and Erwin, 1980; McMurtrey et al., 1977). This suggestion was supported by the findings of Pearson and Turner (1979) that, in vitro, serine hydroxymethyl transferase (SHMT) is capable of forming THBC from tryptamine. This enzyme converts serine into glycine and concomitantly, tetrahydrofolic acid (FH_4) into CH_2=FH_4. As has been postulated recently, an overproduction of CH_2=FH_4, caused by an increased demethylation of serine, could evoke a schizo-affective, mescaline-like psychosis. This type of acute psychosis is characterized at the onset by multiple sensory perceptual distortions, hallucinations, affective symptoms and finally culminating into catatonic states. The typical hallucinogenic drug-like symptoms could be evoked by oral administration of serine and in a few cases by glycine in a dose of 2 mmol/kg or by the consumption of food containing large amounts of serine and glycine. In 20 % of these patients also porphyria was detected. This supports our hypothesis, since the high demand for glycine during a porphyric attack will be met by demethylation of serine by the action of SHMT (Pepplinkhuizen et al., 1980; Bruinvels et al., 1980). As pointed out above, the concomitantly formed CH_2=FH_4 could be responsible for the production of tetrahydro-beta-carbolines (THBC) or tetrahydroisoquinolines (THIQ) via the Pictet-Spengler reaction.

The present study was undertaken to investigate the possibility of an animal model in order to obtain more information about the postulate that an increased production of CH_2=FH_4 during increased conversion of serine into glycine, may be responsible

for the induction of psychosis.

METHODS

A total number of 54 male albino Wistar rats (125-150 g) was used in the present study. Per experiment rats were divided into 3 groups of three rats. Six experiments were performed. Rats were starved for 24 h before the injection of the porphyrinogenic agents.

Induction of porphyria. One group of three rats was injected with 25 mg/kg (i.v.) lead acetate (Merck) dissolved in 0.25 ml glass-distilled demineralized water (demidest) and 50 mg/kg (i.p.) phenobarbital-sodium salt (Gist-Brocades) in 0.5 ml 0.9% NaCl (Maxwell and Meyer, 1976). A second group of three rats was injected with 400 mg/kg allylisopropylacetamide (AIA, s.c., a gift from Hoffman LaRoche; Marver et al., 1966) dissolved in 1 ml polyethylene glycol (J.T. Baker Chemicals). A control group of three rats received polyethylene glycol. The rats were placed in metabolic cages (Acme Metal Products, Chic., Ill., USA) immediately after injection of the porphyrinogenic agents for separate collection of urine and faeces.

Administration of serine or glycine. At maximum porphyria, which was 18 h after injection of lead + phenobarbital (Maxwell and Meyer, 1976) and 24 h after injection of AIA (Marver et al., 1966) one rat in each group received serine, glycine (both from Aldrich, 2 mmol/kg in 0.5 ml 0.9% NaCl) or saline, respectively (i.p.).

Catalepsy. Catalepsy was measured once before injection of serine, glycine or saline, and at 5, 10, 20, 30 and 60 minutes after injection of the amino acid. The catalepsy measurements were performed by placing the rat on a vertical grit with a grit size of 1.5 x 1 cm, about 10 cm above the cage floor. The time the animal did not displace one of his front- or hind paws out of this position was recorded. Catalepsy was scored as follows:

time (sec)	score
0	0
0-10	1
10-20	2
20-30	3
>30	4

To detect statistical differences Students' t-test was performed using the total scores of each rat representing the sum of catalepsy scores measured in duplo at 5, 10, 20, 30 and 60 minutes after injection of amino acid.

Excretion of d-ALA and PBG in the urine. delta-Aminolevulinic acid (d-ALA) and porphobilinigen (PBG) in the urine were determined according to Doss and Schmidt (1971) with some modifications. Urine was collected in dark brown collection bottles which were acidified in advance with 100 ul 100% acetic acid in order to keep the urine at a pH below 4.7. For determination of d-ALA and PBG (both from Sigma Chemical Co.), 3 x 0.5 cm ion-exchange columns were used. To extract d-ALA from the urine, 0.55 g cationexchange material (Ag 50 WX4 100-200 mesh, H^+-form, Bio-Rad) was used. PBG was extracted with 0.55 g anionexchanger (Ag 1X8 100-200 mesh, acetate form, Bio-Rad). The columns were rinsed with 10 ml deionized water before use. The thawed urine samples were adjusted between pH 6.0 and 6.5 with solid $NaHCO_3$ just before 0.5 ml of urine was loaded onto each column. The columns were rinsed with 3x10 ml deionized water, and PBG was eluted with 2x2 ml 1M acetic acid, while d-ALA was eluted with 5 + 2 ml 1M Na-acetate. 0.2 ml acetylaceton (Merck) was added to the d-ALA eluates which were incubated for 10 min at 100°C to convert d-ALA into a pyrrolic compound. To detect the pyrrolic compounds 2 ml of each PBG column eluate or each d-ALA incubate was mixed with 2 ml Ehrlich's reagent (100 ml 2.4% (w/v) p-dimethylaminobenzaldehyde in 100% acetic acid mixed with 19 ml 70% $HClO_4$). This mixture was incubated for 15 min at 37°C and extinction was measured at 553 nm.

Determination of serine and glycine plasma levels. Blood samples were taken every 2 min before measurement of catalepsy by cutting a small slice from the tail. 50 ul of blood was taken into heparinized capillaries. The capillaries were centrifuged for 11 min at 12000 rpm (15000xg) in a Haemofuge (Heraeus). Plasma was separated by cutting the capillaries just above the layer of erythrocytes and transferred on a piece of Parafilm and diluted with 4 volumes of elution buffer A as used in HPLC (see below). This mixture was incubated at 4°C for 60 min and solid particles were spun down in a Beckman Microfuge for 5 min at 9000xg. Subsequently 10 ul of supernatant was injected into a Hewlett Packard Liquid Chromatograph 1084 B equipped with a 20 cm LiChrosorb RP-8 (5 um) column (Hewlett Packard). Serine and glycine were determined by using a "Dynamic Ion Exchange" system (Kraak et al., 1977) with some modifications: Eluens A consisted of 0.5% sodiumdodecyl sulphate (SDS, Merck, w/v) 0.5 mM sodiumcitrate, 0.3% t-amylalcohol (v/v) and was adjusted at pH 3.20 with citric acid. Eluens B consisted of 0.5% SDS (w/v), 10 mM Na_3PO_4, 0.3% t-amylalcohol (v/v) and was adjusted to pH 7.9 with H_3PO_4. Serine and glycine were eluted with buffer A at a flow rate of 2.4 ml/min at 40°C, followed by 4 min buffer B and 10 min buffer A at the same flow rate and temperature to elute the slower compounds and those not of interest and to equilibrate the column

for the next analysis, respectively. The amino acids were detected by postcolumn derivatization with o-phthaldialdehyde (OPA, Merck) at ambient temperature using a mixing chamber connected to the end of the analytical column. A Duramat Dosing pump (Chemie & Filter GmbH, Verfahrenstechnik K.G., Heidelberg 1), which was set at a flow rate of 2.4 ml/min was used to pump the OPA-reagent into the mixing chamber. Fluorescence was measured 3 sec afterwards in an Aminco SPF 500 ratio fluorimeter using an excitation wavelength of 337 nm (bandwith 10 nm) and an emission wavelength of 452 nm (bandwith 40 nm). The OPA-reagent was prepared by dissolving 0.5 g OPA in 10 ml ethanol and mixing with 2 ml 2-mercaptoethanol (sol. I). 1 l 3% H_3PO_4 (w/v) was adjusted to pH 10.2 with NaOH pellets (sol. II; Cronin, et al., 1979). Sol I and sol. II were mixed and kept under nitrogen in dark brown bottles. Under these conditions, the reagent was stable for two weeks (Cronin, et al., 1979).

RESULTS

Porphyria. The induction of porphyria was shown by measuring the excretion of d-aminolevulinic acid (d-ALA) and porphobilinogen (PBG) in the urine. In rats made porphyric by the injection of lead + phenobarbital d-ALA excretion per 48 h was about 28x increased as compared to the excretion by non-porphyric control rats, while PBG excretion per 48 hr in these rats was twice the amount of control rats. The AIA pretreated rats showed an opposite pattern: PBG excretion in these animals was about 35x the amount excreted by non-porphyric controls, while d-ALA excretion was increased by a factor 4 as compared to controls (Fig. 1).

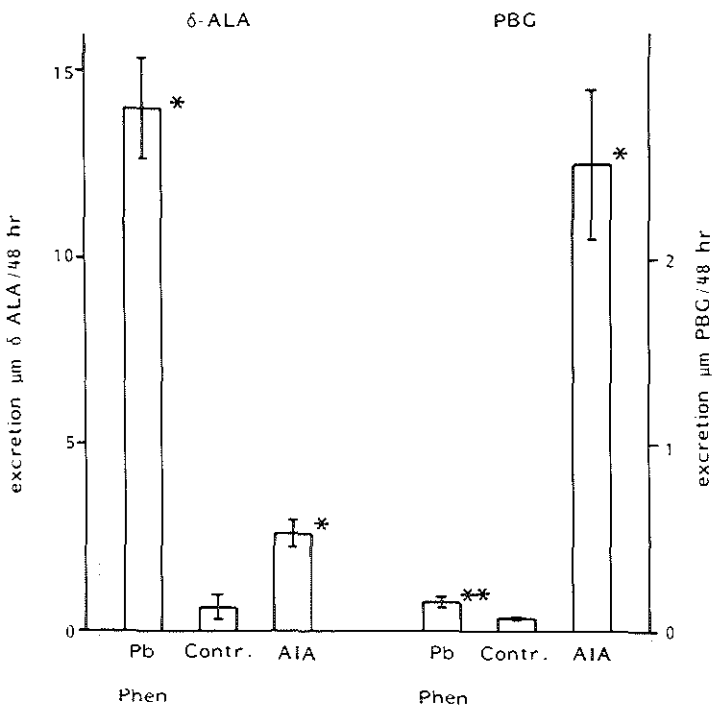

Fig. 1. Excretion of d-aminolevulinic acid (d-ALA) and porphobilinogen (PBG) in the urine during 48 hr after injection of lead + phenobarbital or AIA.
*P<0.001
**P<0.005 (Student t-test).

As shown in Fig. 2, blood serine and glycine levels were

changed in lead + phenobarbital pretreated rats. Plasma serine levels were significantly elevated 18 h after injection of lead + phenobarbital, while glycine levels were significantly lowered. No significant differences in plasma serine and glycine levels were obtained in AIA pretreated animals measured 24 h after injection of AIA.

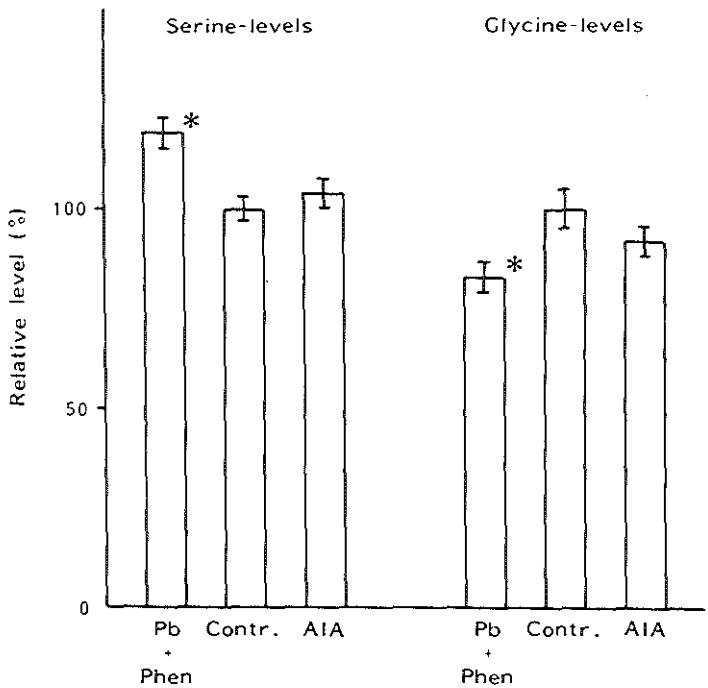

Fig. 2. Serine and glycine blood plasma levels during porphyria. These levels were measured 24 hr after injection of AIA and 18 hr after injection of lead + phenobarbital respectively, and before injection of serine or glycine.
*$P<0.005$ (Student t-test). Control serine and glycine plasma levels were 404 (± 13) umol/l and 527 (± 24) umol/l, respectively.

No changes in urinary excretion of d-ALA and PBG could be found as a consequence of serine or glycine administration in both porphyric and control rats.

As shown in Fig. 3, a negative, semilogarithmic correlation between d-ALA excretion and plasma glycine levels was found using the data obtained from porphyric and control rats before treat-

ment with serine or glycine.

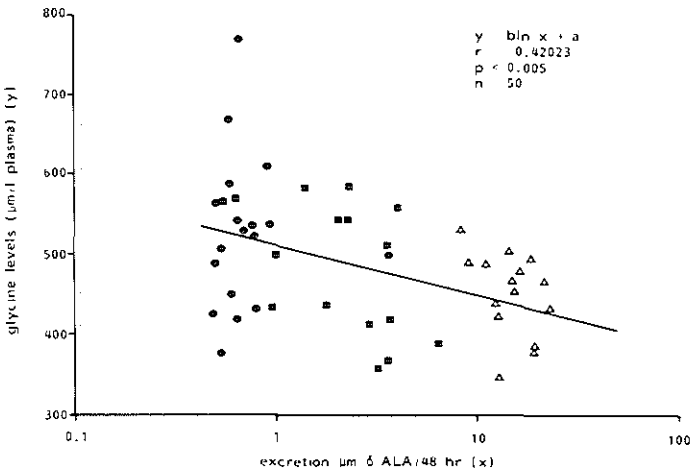

Fig. 3. Semilogarithmic correlation between excretion of d-ALA during 48 hr in the urine and glycine blood plasma levels after pretreatment, measured 24 hr after injection of AIA (⊠) or saline (●) and 18 hr after injection of lead + phenobarbital (Δ).

Serine and glycine administrations. Injection of glycine into lead + phenobarbital pretreated rats did not increase serine plasma levels (Table 1A), while in AIA pretreated animals serine levels were elevated 20-60 min after glycine injections as compared to AIA pretreated, saline injected controls. Only at 30 min after glycine administration to saline pretreated rats significantly elevated serine levels could be found. Comparable results were obtained for the conversion of serine into glycine, indicating no detectable conversion in lead + phenobarbital or saline pretreated rats but an increased conversion in AIA-pretreated rats 20 min after injection of serine (Table 1B).

Catalepsy scores. Administration of serine or glycine to AIA pretreated rats evoked catalepsy after administration of either amino acid as shown in Table 2. However, in lead + phenobarbital pretreated rats, only administration of glycine caused cataleplepsy. No catalepsy was found in rats pretreated with lead + phenobarbital or AIA alone, nor could it be shown after administration of serine or glycine in non-pretreated rats. Most of the cataleptic rats also exhibited widely opened eyelids and stiffness of the tail (Straub tail).

pre-treatment	inj. amino acid	Time in min ± SEM 0	20	30	60
lead + phenob.	serine	100* ± 8.1	175.5 ± 4.9	178.2 ± 8.3	151.7 ± 3.8
	glycine	100 ± 8.4	106.1 ± 6.1	104.7 ± 6.5	103.5 ± 4.2
	saline	100 ± 4.2	90.5 ± 3.0	92.0 ± 2.7	91.8 ± 3.7
AIA	serine	100 ± 8.6	207.3 ± 11.0	190.2 ± 6.5	160.4 ± 4.4
	glycine	100 ± 7.9	103.5$ ± 2.0	103.1$ ± 3.6	98.6$ ± 3.8
	saline	100 ± 3.7	90.9 ± 2.6	85.0 ± 3.0	82.6 ± 2.8
saline	serine	100 ± 11.5	187.0 ± 13.7	183.2 ± 15.1	156.1 ± 11.2
	glycine	100 ± 4.2	100.9 ± 5.1	101.6# ± 4.1	93.5 ± 5.1
	saline	100 ± 5.4	93.7 ± 1.9	89.8 ± 2.1	86.8 ± 2.1

Table 1A. Serine levels in porphyric rats injected with serine or glycine.
* Mean as percent of control level (± S.E.M.) of 6 rats.
$ $p<0.01$ (Student t-test, amino acid injected vs. saline injected in the same pretreated group at indicated time).
# $p<0.05$.

pre-treatment	inj. amino acid	Time in min ± SEM 0	20	30	60
lead + phenob.	serine	100* ± 6.3	92.1 ± 3.3	96.2 ± 3.7	96.3 ± 2.7
	glycine	100 ± 4.3	302.1 ± 32.9	303.5 ± 30.7	218.9 ± 17.9
	saline	100 ± 3.7	93.6 ± 2.6	91.7 ± 1.4	87.0 ± 4.1
AIA	serine	100 ± 6.9	102.0$ ± 3.7	97.2 ± 2.3	102.4 ± 4.9
	glycine	100 ± 8.6	268.4 ± 31.9	229.9 ± 13.6	193.7 ± 6.7
	saline	100 ± 4.0	96.7 ± 1.7	93.2 ± 1.4	96.1 ± 2.0
saline	serine	100 ± 8.2	90.1 ± 2.0	95.0 ± 3.2	100.7 ± 5.2
	glycine	100 ± 7.2	249.2 ± 22.8	239.6 ± 14.5	167.8 ± 6.2
	saline	100 ± 4.5	92.5 ± 5.2	94.4 ± 1.5	91.9 ± 2.2

Table 1B. Glycine levels in porphyric rats injected with serine or glycine.
* Mean as percent of control level (± S.E.M.) of 6 rats.
$ $p<0.02$ (Student t-test, amino acid injected vs. saline injected in the same pretreated group at indicated time).

pretreatment	inj. amino acid	total scores	± SEM
lead + phenobarbital	serine	9.3$	± 1.7
	glycine	16.7**	± 1.6
	saline	11.1	± 3.7
saline	serine	10.0	± 2.8
	glycine	10.0	± 1.6
	saline	8.7	± 1.7
AIA	serine	19.8#	± 1.0
	glycine	17.0*	± 2.6
	saline	9.8	± 2.8

Table 2. Catalepsy scores in porphyric rats.
$ Mean of total catalepsy scores (representing the scores measured at 5, 10, 20, 30, and 60 min after amino acid injection) obtained from 6 rats.
* $p<0.05$ and ** $p<0.02$ vs. glycine injected, non-pretreated rats.
# $p<0.01$ vs. serine injected, non-pretreated rats.
Statistical differences were measured by applying Student's t-test.

DISCUSSION

To enhance the demand for glycine, porphyria was chemically induced in rats, which were starved 1 day before treatment. For this purpose, injection of 2-allyl-2-isopropylacetamide (AIA) was used as one method and administration of lead + phenobarbital as the other (Marver et al., 1966; Maxwell and Meyer, 1977; Unseld and De Matteis, 1978).

Administration of AIA resulted in massive excretion of PBG per 48 h in the urine. AIA quite specifically acts as a porphyrinogenic agent by accelerating the breakdown of heme, which is the feedback inhibitor of the heme pathway, and heme proteins, thereby inducing the activity of d-aminolevulinic acid synthetase (d-ALAS; Marver et al., 1966; Unseld and De Matteis, 1978; Stephens et al., 1978). This enzyme will produce large amounts of d-ALA, which is quickly converted into porphobilinogen (PBG, Marver and Schmid, 1972). In order to meet this massive production of d-ALA glycine synthesis has to be increased since the latter is one of the two substrate for d-ALAS.

Both lead and phenobarbital are slightly porphyrinogenic. However, when injected simultaneously, they have a strong potentiating effect (Maxwell and Meyer, 1976). Our results revealed a 28x increase of d-ALA excretion in the urine during 48 hr, indicating the induction of d-ALAS, which is in agreement with the findings of Maxwell and Meyer (1976).

The present results show that glycine levels are correlated with d-ALA excretion into the urine in both types of porphyria (Fig. 3), probably because the demand for glycine is enhanced. Because most of the glycine pool is synthesized from serine via serine hydroxymethyltransferase (SHMT; Nyhan, 1972; Arnstein, 1954), increased serine levels in lead + phenobarbital pretreated rats are not expected to occur together with the enhanced need for glycine in porphyria. Probably SHMT is inhibited by lead, which can react with the sulfhydryl groups at the active site of SHMT (Moore et al., 1980; Schirch et al., 1980). This would explain both the lowered glycine levels and increased serine levels in lead + phenobarbital pretreated rats.

Different types of animal behaviour can be used as an indication for psychotic behaviour (Maxwell, 1968; Smythies et al., 1969; Bloom et al., 1972; Fisher, 1975). Among these, catalepsy and catatonia are the most frequently used (Maxwell, 1968; Bloom et al., 1972; Segal et al., 1977). Although these terms are often

used interchangeable, they have dissimilar behavioural profiles and may imply different categories of participating neurotransmitter systems (Costall and Naylor, 1974). The difference between the rigid- and non-rigid-type of immobility (catatonia and catalepsy, respectively) is based on the findings obtained with opiate-like drugs and the behaviour seen after neuroleptic drug treatment. According to Segal et al. (1977), the vertical grid test, as used in the present study, measures catalepsy. However, some of our cataleptic rats also showed stiff tails and wide-opened eyelids, which is suggestive for catatonia (Segal et al., 1977). Therefore, both serine and glycine probably evoked cataleptic as well as catatonic behaviour, which may be comparable to the catatonic behaviour shown by some of our patients (Pepplinkhuizen et al., 1980).

The observation that serine, when injected into lead + phenobarbital pretreated rats could not evoke catalepsy, while glycine did, is in agreement with the proposed inhibition of SHMT by lead as discussed above. This would also explain the increased glycine plasma levels in lead + phenobarbital pretreated, glycine injected animals (Table 1B).

The finding that glycine can evoke catalepsy would not appear to fit in with our hypothesis that the elevated conversion of serine into glycine gives rise to high levels of CH_2=FH_4. However, high amounts of glycine can be metabolized in the glycine cleavage system (GCS) to CO_2 and NH_3, concomitantly forming CH_2=FH_4 from FH_4. As pointed out above, increased levels of CH_2=FH_4 could be intermediates in alkaloid production.

Tetrahydro-beta-carbolines (THBC) and tetrahydroisoquinolines (THIQ) can be formed from indoleamines and catecholamines respectively and formaldehyde, formed by decomposition of CH_2=FH_4. The possibility that formaldehyde-derived biogenic amines can act as psychotogenic or "schizophrenic" agents, is suggested by many authors (Deitrich and Erwin, 1980; Airaksinen and Kari, 1981 I; Sandler et al., 1973). Especially THBC's, which are derived from indoleamines, received much attention, as reviewed by Airaksinen and Kari (1981 I and II) and Airaksinen and Mikkonen, (1980).

Studies are in progress to isolate and identify the compounds responsible for inducing catalepsy in rats. These compounds may also be responsible for evoking episodic psychosis in patients.

In conclusion, catalepsy could be evoked during experimental

porphyria in rats after injection of serine and glycine, and may probably represent a good model for studying a schizophrenic-like psychosis as described previously (Pepplinkhuizen et al., 1980; Bruinvels et al., 1980). The AIA-induced porphyria seems to be more suitable for further experimentation, because this drug acts in a more specific way on the heme pathway.

Acknowledgements

In these studies we were supported by the excellent technical assistance of A. Edixhoven and Y. Tjoa, and the fruitful discussions with Dr. L. Pepplinkhuizen and Dr. P. Moleman.

CHAPTER III

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF TETRAHYDRO-BETA-CARBOLINES EXTRACTED FROM PLASMA AND PLATELETS

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by

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Summary

A fast method for extraction and concentration of tryptamine (TA), 5-hydroxy-TA and 5-methoxy-TA was developed using reverse phase C-18 sample preparation columns in combination with an ion-pairing reagent. Using this method, 1,2,3,4-tetrahydro-beta-carboline (THBC), 6-hydroxy-THBC and 6-methoxy-THBC, the respective reaction products formed after reaction of formaldehyde with the primary amines mentioned above, and beta-carboline (BC, norharman) and 1-methyl-beta-carboline (1-Me-BC, harman) could be extracted from human and rat platelets and platelet poor plasma (PPP). A HPLC method combined with fluorometric detection was developed for the quantitative determination of these compounds in the picomole range. The formation of beta-carbolines during the extraction procedure was below the limit of detection of the assay procedure. 6-OH-THBC, THBC, 1-Me-BC and 5-HT were identified as normal constituents of human platelets, whereas only 5-HT and 6-OH-THBC could be identified in human PPP. In rat platelets and PPP 5-HT, but no THBC's could be detected.

INTRODUCTION

During the last two decades interest has arisen in the hypothesis that endogenously formed 1,2,3,4-tetrahydro-beta-carbolines (THBC's) could contribute to psychotic events observed in alcoholism or in schizophrenia (Bruinvels et al., 1980; Airaksinen and Kari, 1981b; Deitrich and Erwin, 1980; Schouten et al., 1983; Pepplinkhuizen et al., 1980). Several methods have been developed for the determination of beta-carboline compounds in brain, adrenal gland, blood platelets and plasma (Bosin et al., 1983; Rommelspacher et al., 1982; Beck et al., 1982b; Barker, 1982; Faull et al., 1982; Kari et al., 1979; Airaksinen and Kari, 1981 I). However, some doubt still exists to their physiological occurance (Bosin et al., 1983, Bloom and Barchas, 1982), because these compounds are very easily formed in vitro under physiological conditions from endogenous tryptamines reacting with formaldehyde or with acetaldehyde by the Pictet-Spengler condensation (Whaley and Govindachari, 1951). Recently, Bosin et al. (1983) demonstrated that THBC's could be formed during sample preparation from formaldehyde present in the organic solvents used in the extraction procedure.

The aim of the present paper was to develop a new method using reverse phase C-18 sample preparation cartridges for sample preparation together with an ion-pairing reagent in order to minimize the use of organic solvents, thus preventing the artifactual formation of THBC compounds. The optimal conditions for extraction of THBC's and their monoamine precursors from human and rat plasma and from platelets, as well as the absence of the artifactual formation of THBC's are described. A new HPLC method was developed to separate and quantify the extracted compounds.

MATERIALS AND METHODS

Materials. 5-hydroxy-tryptamine.HCl (5-HT.HCl) and 1-methyl-beta-carboline (1-Me-BC, harman) were obtained from Sigma Chemical Co., 5-methoxy-tryptamine.HCl (5-MeO-TA.HCl) from Aldrich, and tryptamine.HCl (TA, purum) and beta-carboline (BC, norharman) from Fluka. The organic solvents were obtained from Merck and were of Uvasol quality. 1,2,3,4-Tetrahydro-beta-carboline (THBC), 6-hydroxy-THBC and 6-methoxy-THBC were synthesized according to the method described by Vejdelek et al. (1961). Reverse phase Sep-pak C-18 cartridges were obtained from Waters Ass.. Zorbax BP TM C8 was purchased from Chrompack (The Netherlands). Sodium octyl sulphate (SOS) was obtained from Merck. Demineralized distilled water (demidest) was used as a solvent.

Sample treatment. Venous blood (30 ml samples) was collected in the morning from 5 healthy volunteers (4 males - 1 female; age range 24-32 years) in 10 ml Vacutainer Thrombotect tubes containing 2.5% EDTA, 0.025% 2-chloroadenosine and 7.0% procaine-HCl, to inhibit platelet aggregation. Platelet rich plasma (PRP, 15 ml) was obtained by centrifuging the samples for 20 min. at 130xg, followed by two 10 min. runs at 450xg and 1400xg (20°C), respectively, and pooling the supernatants. Platelets and platelet poor plasma (PPP) were separated by centrifuging the PRP for 10 min. at 10000xg and 10°C.

Rat blood samples (10 ml) were collected from 3 male Wistar rats (275- 300 g) by heart puncture (under light ether anesthesia) using a 0.7 x 32 syringe needle which was heparinized to prevent blood clotting. To obtain the PRP, these samples were centrifuged for 20 min. at 200xg and 20°C followed by two 15 min. runs at 600xg and 1400xg, respectively (both at 20°C). The pooled supernatants (PRP, 5ml) were centrifuged for 10 min. at 10000xg and 10°C to obtain platelets and PPP. The number of platelets/liter PRP was determined by electronic counting in a Coulter Counter Model ZF.

Extraction procedure. The extraction efficiency of the C-18 cartridges was optimal at an elution flow rate of 2 ml/min. An Ismatec MP-25 25-channel peristaltic pump connected with 20 ml syringes which were fitted upon the C-18 cartridges, was used to compress the air above the samples admitted to the cartridges, in order to obtain reproducible flow rates. The cartridges were rinsed with 5 ml methanol, followed by 5 ml demineralized, glass-distilled water (demidest), and finally with 5 ml extraction buffer. This buffer contained 60 mM $NaH_2PO_4.2H_2O$, 0.1 mM EDTA (di Na-salt), 5 mM semicarbazide, 0.5 mM ascorbic acid and 0.8 M $NaClO_4$ and was adjusted to pH 4.0. with H_3PO_4. Ten ml extraction buffer was added to the platelet pellets (see Sample treatment)

which were sonicated using a MSE 150 watt ultrasonic desintegrator (20 KHz) for 30 sec at 20 micron amplitude. Remaining membrane fractions were spun down for 10 min. at 10000xg and 20°C, and the supernatant was loaded onto the Sep-pak cartridge. One volume of 5x concentrated extraction buffer was added to 4 volumes of platelet poor plasma (PPP, see Sample treatment) and loaded onto the cartridges. The cartridges were rinsed with another 5 ml extraction buffer and eluted with 3 ml methanol. The extracts were concentrated by evaporation in a Rotavapor (Buchi), and the residue was taken up in 1.0 ml 25 mM $(NH_4)_2HPO_4$ buffer (pH 4.0) containing 10 mM sodium octylsulphate (SOS), 0.5 mM EDTA, 25 mM semicarbazide and 2.5 mM ascorbic acid.

Recoveries from the Sep-Pak columns were determined by carrying out the described extraction procedure using 10 ml extraction buffer containing 5 nmol of each compound (Fig. 3). Recoveries from PRP and platelet pellets were determinded according to the same procedure, using the sample volumes as described in the preceding section.

Chromatographic procedure. High performance liquid chromatography was performed on a HP 1084 B Liquid Chromatograph (Hewlett Packard) using a stainless steel column (250 x 4.6 mm) home-packed with Zorbax BP TM C8 (7-8 micrometer; Chrompack). A Chrompack 75 x 2.1 mm Guard Column was used to protect the analytical column. The flow was kept at 2 ml/min at a temperature of 45°C.

System I. With this chromatographic system 6 aromatic amines could be separated: Tryptamine (TA), 5-hydroxy-TA (5-HT), 5-methoxy-TA (5-MeO-TA), 1,2,3,4-tetrahydro-beta-carboline (THBC), 6-Hydroxy-THBC (6-OH-THBC) and 6-methoxy-THBC (6-MeO-THBC). Buffer A, containing 1 mM SOS, 25 mM $(NH_4)_2HPO_4$ and 0.5 % 2-propanol (Merck, Uvasol), was dissolved in demidest, adjusted to pH 8.0 and filtered through a 0.45 micrometer HA filter (Millipore). Buffer B, consisting of 1 mM SOS, 50 mM $(NH_4)_2HPO_4$, and 25 % 2-propanol was adjusted to pH 6.0 and filtered through a 0.5 micrometer FH filter. A gradient system was used as depicted in Fig. 1. Injectionvolume was 200 microliter. The compounds were detected by using an Aminco SPF 500 ratio fluorometer fitted with a 35 microliter flow cell. Excitation wavelength was 290 nm (bandwith 15 nm) and emission wavelength was 335 nm (bandwith 40 nm).

System II. With this system beta-carboline (BC, norharman) and 1-methyl-BC (1-Me-BC, harman) could be separated. Chromatography was performed with a single buffer system containing 25 mM $(NH_4)_2HPO_4$ and 30 % 2-propanol in demidest. The pH was adjusted to pH 6.0. Injection volume, buffer flow rate and column temperature were the same as described above. However, no gradient system was needed to separate BC and 1-Me-BC. In this system detection was performed by measuring the fluorescence using an

excitation wavelength of 375 nm (bandwith 15 nm) and an emission wavelength of 434 nm (bandwith 40 nm).

Quantitation. Quantitation of the eluted peaks was performed by automatic peak area integration using the Hewlett Packard 79850B terminal. When quantitating small peaks, the use of the integrator is limited by the lowest detectable slope increase in relation to the baseline noise. Under these circumstances we used peak height as a measure for the concentration of 6-OH-THBC, THBC and 1-Me-BC, isolated from platelets or plasma. The latter method proved to be more accurate then electronic integration for peak heights ranging from 3-15x baseline noise. The precision of this method depended on the peak height measured, and ranged from 89% (peak height 3x baseline noise) to 98% (peak height 15x baseline noise). Quantitation was performed using external standards. Retention times were automatically printed in hundreds of minutes.

RESULTS

Chromatography. HPLC-separation of a mixture of beta-carbolines and their precursors using a Na_2HPO_4 buffered system without the addition of an ion-pairing reagent resulted in bad separations of the indole- and beta-carboline compounds and broad, tailing peaks (Fig.1A). Addition of the ion-pairing reagent sodium octylsulphate (SOS) to a Na_2HPO_4 buffered chromatographic system significantly increased retention times, while peak shapes

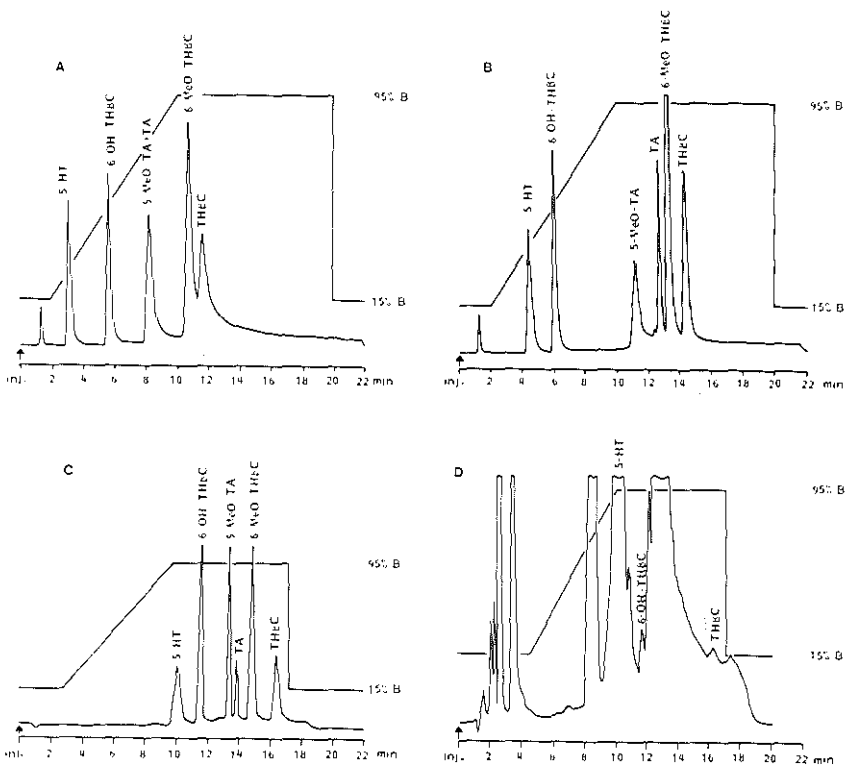

Fig. 1. HPLC of indoleamine and beta-carboline compounds. Flow: 2 ml/min.; Temp. 45°C.; 1A: Standard sample (200 pmol/compound). Buffer A: 25 mM Na_2HPO_4, 0.5 % 2-propanol, pH 8.0; buffer B: 25 mM Na_2HPO_4, 25 % 2-propanol, pH 6.0; 1B: Standard sample; buffers as in Fig 1A, with the addition of 1 mM sodium octylsulphate (SOS) in both buffers; 1C: Standard sample; System I (see Methods); Fig 1D: Human platelet sample, system I.

greatly improved (Fig. 1B). Replacement of Na^+ by NH_4^+-ions (=System I; Fig. 1C) resulted in a further improvement of peak shapes (System I). A pH- and propanol gradient was necessary to optimize the separation of these compounds. Buffer A, adjusted to pH 8.0 proved to be optimal for the separation of 5-HT and 6-OH-THBC, whereas buffer B, adjusted to pH 6.0 was optimal for separation of the other four aromatic amines. Under these conditions neither BC nor 1-Me-BC were detectable, as a result of different chromatographic and fluorometric properties (see description System II).

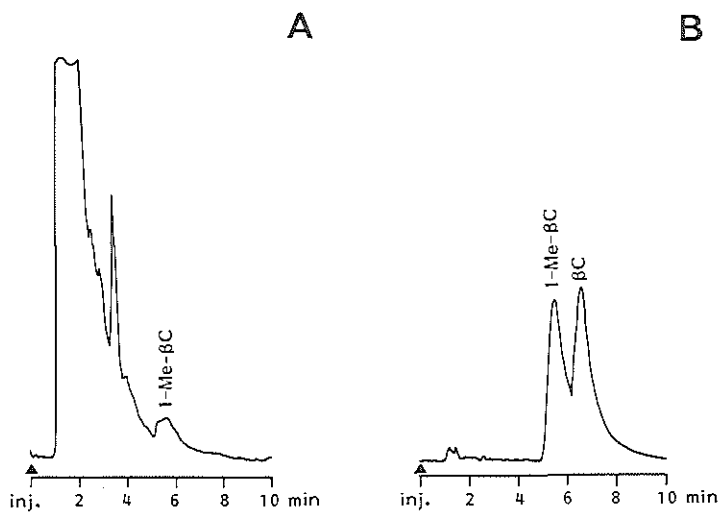

Fig. 2. HPLC of BC and 1-Me-BC. (System II, see methods) Flow: 2 ml/min; Temp. 45°C. **2A:** Human platelet extract; **2B:** Standard sample, 200 pmol/compound.

Separation of 1-Me-BC and BC could be partly achieved by using a single buffer system (system II, pH 6.0) on the same column (Fig. 2A). For optimal separation of these two compounds the pH had to be adjusted to 6.0. Addition of SOS to System II resulted in bad separations and increased retention times (chromatogram not shown). Under these conditions none of the THBC's and indoles, as measured in System I, were detectable.

Extraction. Increasing concentrations of $NaClO_4$ in the extraction buffer, using the Sep-Pak C-18 extraction procedure significantly improved recovery of 5-HT and 6-OH-THBC (Fig. 3A), while the optimal pH for extraction ranged from 4.0 to 6.0 (Fig. 3B). The lower pH limit (pH 4.0) was chosen in the standard

procedure in order to minimize in vitro formation of beta-carboline compounds (Whaley and Govindachari, 1951). Under these conditions extraction recoveries of 5 nmol 1-Me-BC and 5 nmol BC were 93 % and 97 %, respectively. In the presence of platelets or PPP recoveries were from 75% for the more hydrophilic compounds (5-HT and 6-OH-THBC) and 94% for the more lipophilic compounds (5-MeO-TA, TA, 6-MeO-THBC, THBC, BC and 1-Me-BC).

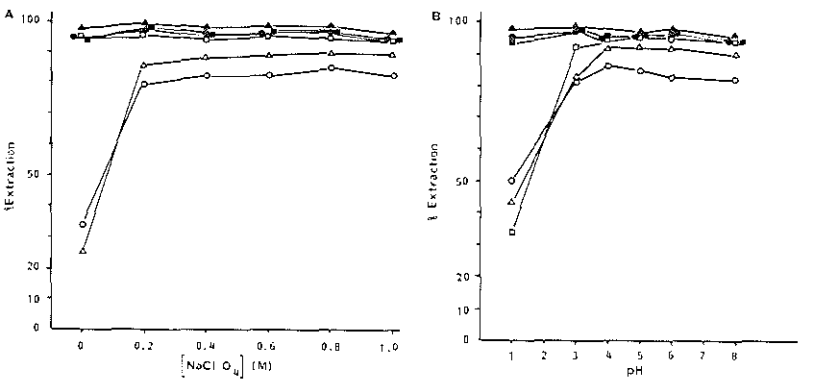

Fig. 3 A: % Extraction vs. [$NaClO_4$] (pH= 5.0). **B:** % Extraction vs. pH ([$NaClO_4$]= 0.6 M). ○-○ 5-HT; △-△ 6-OH-THBC; □-□ 5-MeO-TA; ●-● TA; ▲-▲ 6-MeO-THBC; ■-■ THBC. These experiments were performed using 5 nmol of each compound tested. Further conditions as described in Materials and Methods.

Formation of beta-carbolines during extraction procedure. Artifactual formation of THBC compounds was checked by spiking platelet and PPP samples with 5 nmol TA, 5-MeO-TA and 5-HT, respectively. After these samples had gone through the entire procedure artifactual formation of THBC's was below limits of detection (see Table 1). However, when semicarbazide was omitted from the extraction buffer or from the extracted samples prior to HPLC analysis, artifactual formation of 6-MeO-THBC from 5 nmol 5-MeO-TA /5 ml PPP could be detected in rat PPP samples (9 pmol 6-MeO-THBC/ml extract). Formation of 6-OH-THBC or THBC from 5 nmol 5-HT or TA, respectively, was below the limits of detection.

Beta-carboline and indoleamine levels in plasma and platelets. Chromatograms of human platelet extracts using system I and II are shown in Fig. 1D and 2A, respectively. 5-HT, but no beta-carbolines could be detected in rat plasma and platelets (Table 1). 6-OH-THBC, THBC, 1-Me-BC and 5-HT could be detected in human platelets, whilst only 6-OH-THBC and 5-HT were found in human PPP (Table 1). TA and 5-MeO-TA levels in human and rat

platelets were less than 0.4 and 0.2 pmol/10^8 platelets, respectively. Human PRP contained 249 ± 40 x10^9 platelets/l PRP, whereas rat PRP contained 1320 ± 123 x10^9 platelets/l PRP. The total number of platelets in the pellets obtained from 15 ml human PRP and 5 ml rat PRP, were 3.7 ± 0.6 x10^9 and 6.6 ± 0.7 x10^9 platelets, respectively.

	platelets (pmol/10^8 ± SEM)		PPP (pmol/ml ± SEM)	
compound	RAT *	HUMAN +	RAT #	HUMAN $
5-HT	627 ± 45	330 ± 54	625 ± 44	160 ± 9
6-OH-THBC	<0.8	5.3 ± 1.1	<4.6	7.4 ± 0.4
6-MeO-THBC	<0.2	<0.2	<2.0	<0.7
THBC	<0.4	2.3 ± 0.3	<4.3	<1.4
1-Me-BC	<0.04	0.26 ± 0.05	<0.5	<0.3
BC	<0.07	<0.1	<0.5	<0.3

Table 1. 5-HT and beta-carbolines in man and rat. The following amounts of sample were used:
* 6.6*10^9 rat platelets/sample;
\+ 3.7*10^9 human platelets/sample;
\# 5 ml rat PPP/sample;
$ 15 ml human PPP/sample. Data were not corrected for recovery.

	retention times (min. ± S.D.)				
		PPP		platelets	
compound	standard	not spiked	spiked	not spiked	spiked
6-OH-THBC	11.63 ± 0.08	11.69	11.58	11.65	11.60
THBC	16.38 ± 0.08	n.d.*	n.s.#	16.33	16.38
1-Me-BC	5.86 ± 0.11	n.d.	n.s.	5.86 ± 0.16	n.s.

Table 2. HPLC retention times of beta-carbolines in human PPP and platelet extracts before and after spiking. Samples were spiked with approximately the same amount of 6-OH-THBC or THBC as found in the samples before spiking. 1-Me-BC was not spiked, but retention times of 1-Me-BC peaks in human platelet extract chromatograms were compared with standard retention times.
* n.d.=not detected.
\# n.s.=not spiked.

To confirm the identity of the beta-carboline compounds measured in plasma and platelets, some human samples were spiked with 6-OH-THBC and THBC (Table 2). The retention times of the detected beta-carbolines in PPP and platelet samples were within the error range of standard retection times (Table 2).

DISCUSSION

Several techniques have been applied to the separation and quantification of beta-carboline derivatives, e.g. high performance liquid chromatography (HPLC) in combination with amperometry (Rommelspacher et al., 1982), thin layer chromatography (TLC) in combination with fluorometry (Rommelspacher et al., 1982) or (^{3}H)-acetic anhydride labeling (Rommelspacher et al., 1979) and, most frequently, gas chromatography (GC) in combination with mass spectrometry (MS, Bosin et al., 1983; Beck et al., 1982 a and b; Barker, 1982; Faull et al., 1982; Kari et al., 1979). This topic has been extensively reviewed by Airaksinen and Kari (1981 I). The GC/MS technique involves derivatization of the beta-carboline compounds with pentafluoropropionyl (PFP) or heptafluorobutyryl (HFB), in order to make the beta-carbolines more volatile. If trace amounts of formaldehyde are present during these derivatization reactions, the condensation reaction between formaldehyde and biogenic amines could easily take place. Another disadvantage of the GC technique in trace analyses involves the need to saturate the GC column with these derivatives prior to quantification of the beta-carboline compounds (Faull et al., 1982). To avoid these problems we investigated the possibility analyzing underivatized beta-carbolines using the HPLC technique.

Chromatography of alkaloid compounds using reverse phase packing materials results in tailing peaks (Fig. 1A and 1B), probably due to the interaction of the aromatic amines with non-silanized Si-OH groups at the packing surface which disturbs the reverse phase separation process (Sokolowski and Wahlund, 1980). These interactions can be prevented by using "end-capped" packing materials, or addition of alkylammonium ions, in order to reduce peak tailing (Sokolowski and Wahlund, 1980). Ammonia was used to shield residual Si-OH groups from interaction with the alkaloid components in the mobile phase.

The addition of sodium octyl sulphate (SOS), also improved peak shape, which may be ascribed to the ionic interaction ("ion-pairing") between SOS and the aromatic amines which prevents the latter from reacting with free Si-OH groups (Olieman et al., 1977). Separation of 1-Me-BC and BC did not improve after addition of SOS.

In order to obtain detectable amounts of beta-carbolines using the present chromatographic system, concentration and clean-up of the samples was necessary. Initial experiments using XAD-2 resin (300-1000 micrometer), 50 WX 8 cation exchange resin

and Sep-pak C-18 reverse phase cartridges showed the latter to be superior in loading capacity. Because small volumes are needed for quantitative elution of the beta-carbolines, only small amounts of organic solvent are required in this procedure. However, 5-HT and 6-OH-THBC, the more hydrophylic aromatic amines, had lower extraction recoveries (28 % and 36 % respectively) due to a lower affinity of these compounds for the Sep-Pak columns (Fig. 3A). The higher extraction recoveries for these compounds using the same columns, reported by Faull et al. (1982), were probably due to the use of smaller sample volumes and smaller volumes for flushing and elution.

Addition of $NaClO_4$ as an ion-pairing reagent dramatically increased the extraction recoveries of 5-HT and 6-OH-THBC (Fig. 3A), whereas the recoveries of the other beta-carbolines remained unaffected. Using this method, it is possible to precipitate proteins with 0.4 - 0.6 M $HClO_4$ prior to the extraction procedure, although this is not a nessecary step to obtain satisfactory extraction recoveries from plasma or platelets.

The optimal pH during extraction ranged from 4.0 to 6.0 (Fig. 3B). because beta-carbolines are more easily formed from indolamines and formaldehyde at alkaline pH (Whaley and Govindachari, 1951), the use of lower pH values (pH 4.0) during extraction reduced the possibility of artifactual formation of these compounds. However, the use of a formaldehyde trapping agent remained necessary. The formation of a small amount of 6-MeO-THBC in rat PPP when semicarbazide was omitted from the extraction buffer in the presence of 5-HT, 5-MeO-TA and TA indicates that 5-MeO-TA reacts more readily with formaldehyde than 5-HT or TA. These results are in accordance with the findings by Bosin et al. (1983). Spiking platelet suspensions or platelet poor plasma (PPP) with 5 nmol 5-MeO-TA in the presence of semicarbazide did not result in artifactual formation of 6-MeO-THBC. It seemes unlikely therefore that the 6-OH-THBC detected in human platelets had been formed from 5-HT during work-up, especially since 5-HT reacts more slowly with formaldehyde than 5-MeO-TA.

Retention times of the beta-carbolines extracted from plasma and platelets were within the range of retention times of standard compounds. In order to confirm the identity of the measured peaks some of these extracts were spiked with a small amount of standard beta-carbolines, and then reanalyzed on HPLC. Beta-carboline retention times after spiking corresponded with retention times before spiking (Table 2), indicating that the measured peaks almost certainly represent the indicated beta-carbolines. Moreover, fluoresence of the eluted peaks were measured using an

excitation wavelength of 290 nm and an emission wavelength of 335 nm for identification and quantification of THBC's, and 375 nm and 434 nm respectively for the unsaturated beta-carbolines. The fluorescence measurement, combined with the use of narrow bandwiths (15 and 40 nm for excitation and emission respectively) also added to the specificity of the detection.

We have compared values of beta-carbolines given in this paper with those previously published. Rommelspacher et al. (1979, 1982) reported that 6-OH-THBC was present in rat and human platelets. We have comfirmed this observation using human platelets, but were unable to detect 6-OH-THBC in rat platelets. We could not detect 6-MeO-THBC using this technique although Barker reported that this compound was present in human platelets (Barker, 1982). THBC in human platelets was detected by several authors at somewhat varying concentrations, but in the same order of magnitude as reported in this paper. We were not however, able

compound	species	locus	conc.	dimension	reference	
5-HT	rat	platelets	229[b]	pm/10^8	Udenfriend	1964
	man	platelets	540[b]	,,	Rommelsp.	1982
	,,	,,	74 - 440	,,	Girard	1963
	,,	PPP	5.7 - 142.9	pm/ml	Crawford	1965
6-OH-THBC	rat	platelets	3.2 - 6.9	pm/10^8	Rommelsp.	1982
	,,	,,	275 ± 129	,,	Rommelsp.	1979
	man	,,	2.7 ± 0.3	,,	Rommelsp.	1982
6-MeO-THBC	man	,,	7.5*,#	,,	Barker	1982
THBC	,,	,,	5.8*	,,	Honecker	1978b
	,,	,,	10.6* ± 1.6	,,	Kari	1979
	,,	,,	0.5*	,,	Honecker	1980
	,,	,,	20*,#	,,	Barker	1982
	,,	plasma	31.4 ± 5.1	pm/ml	Kari	1979
1-Me-BC	man	platelets	detected	-	Bidder	1979

Table 3. Beta-carbolines in man and rat found by other workers. All results from other reports are expressed as pmol/10^8 platelets or as pmol/ml PPP, in some cases with standard deviation.
* Data were obtained by recalculation of original data.
\# In this study THBC (10.3 ng/ml) and 6-MeO-THBC (3.8 ng/ml) were identified in whole blood. The amount of beta-carboline/10^8 platelets was calculated assuming that 1 ml of whole blood contains $3x10^8$ platelets and all beta-carbolines are concentrated into the platelets.

to detect THBC in human PPP, as reported by Kari et al. (1979). The concentration of 5-HT in human plasma and platelets using our method is in agreement with values reported by others (Udenfriend, 1964).

The present 5-HT values in rat plasma are quite high. Since 5-HT in human plasma was in the normal range, these high values in rat plasma could have been due to a higher incidence of platelet disruption since, in this species, the number of platelets per ml is 4x higher than in human PRP, while 5-HT content in rat platelets is about the same (Garattini and Valzelli, 1965; Altman and Dittmer, 1964).

In conclusion, the presently described procedure is a fast and simple method for extraction, concentration and quantification of beta-carboline derivatives and their precursors, although additional identification may be necessary by using another more selective detection scheme, like mass spectrometry (experiments in progress). The values of the beta-carbolines found in blood platelets and PPP are in accordance with or less then concentrations reported by others. The failure to detect these substances using the present method cannot be due to a lower sensitivity, but may rather suggest that they were artifactually formed during the isolation and analytical procedures employed by others.

Acknowledgements

In these studies we were supported by the excellent technical assistance of Y. Dikkerboom and T. Adolfs. We also acknowledge G. Elliott for careful reading the manuscript.

CHAPTER IV

ENDOGENOUSLY FORMED NORHARMAN (beta-CARBOLINE) IN PLATELET RICH PLASMA OBTAINED FROM PORPHYRIC RATS

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by

M. Joris Schouten and Jacques Bruinvels

Summary

Porphyria was induced in adult male Wistar rats starved for 24 hrs by s.c. injection of 400 mg/kg allylisopropylacetamide (AIA). The presence of porphyria was shown by measuring excretion of delta-aminolevulinic acid (delta-ALA) and porphobilinogen (PBG) into the urine during 24 hr after AIA administration. Plasma levels of glycine, serine and of a number of other amino acids were decreased in porphyric rats as compared to controls. Intraperitoneal injection of 2 mmol/kg serine 24 hr after AIA administration was used as an animal model for an acute psychosis, by measuring catalepsy scores 30 min after serine injection. The concentration of 5 different beta-carbolines in platelet rich plasma (PRP) was measured using an HPLC-fluorometric method. An increase in the concentration of norharman (NH) in PRP, ranging from 0.57 nmoles/l in control rats to 1.88 nmoles/l in serine treated porphyric rats was found. The catalepsy duration was exponentially correlated with the NH concentrations in PRP. It is concluded that an elevated conversion of serine into glycine via serine hydroxymethyltransferase (SHMT) may be responsible for the enhanced NH biosynthesis.

INTRODUCTION

Initiated by the transmethylation hypothesis of Osmond and Smythies (1952), several investigators have postulated the endogenous formation of beta-carbolines from indoleamines acting as "psychotogens" (Bruinvels and Pepplinkhuizen, 1984; Honecker et al., 1980; Kari et al., 1979; Schouten and Bruinvels, 1983). However, until now, no increased levels of beta-carbolines in schizophrenics could be detected (Rimon et al., 1984). The involvement of beta-carbolines in a group of patients suffering from an episodic, schizo-affective psychosis of the manic-psychedelic type has been suggested previously. It was shown that within 2-3 hours after oral administration of serine to clinically recovered psychotic patients, psychotic symptoms reappeared (Pepplinkhuizen et al., 1980; Bruinvels et al., 1980). An increased conversion of serine into glycine was proposed to be responsible for the production of beta-carbolines, thus evoking psychotic symptoms.

It has been shown previously that i.p. injection of serine into porphyric rats causes catalepsy. This behavioural phenomenon was used as an animal model for the acute psychosis studied (Schouten and Bruinvels, 1983). According to the hypothesis described above,it was proposed that in porphyric rats beta-carbolines could be formed as a result of an increased serine to glycine conversion via the enzyme serine hydroxymethyltransferase (SHMT). Pearson and Turner (1979) showed that 1,2,3,4-tetrahydro-beta-carboline (THBC) can be formed in vitro by SHMT if incubated with serine in the presence of tryptamine. This finding supports our hypothesis that an increased conversion of serine into glycine in vivo could give rise to the endogenous formation of beta-carbolines.

The aim of the present study was to investigate whether an increased formation of beta-carbolines occurred in porphyric rats treated with serine, and whether this is related to the cataleptic behaviour observed.

MATERIALS AND METHODS

Male Wistar rats (280-320 g) were starved for 48 hr. After 24 hr the starving rats were injected subcutaneously with 400 mg/kg allylisopropylacetamide (AIA, a generous gift of Hoffmann-La Roche, The Netherlands), dissolved in 1 ml polyethyleneglycol (PEG), which had an approximate molecular weight of 200 (Baker Grade, Baker Chemicals). During the following 24 hr of starvation urines were collected in dark brown glass bottles to which 0.1 ml glacial acetic acid (Merck) was added before collection of the urine, in order to prevent delta-aminolevulinic acid (delta-ALA) breakdown. delta-ALA and porphobilinogen (PBG) were determined as described previously (Schouten and Bruinvels, 1983). After the 48 hr starvation period a blood sample of 0.4 ml was taken by heart puncture under light ether anaesthesia just before the administration of serine, for determination of plasma amino acids. Amino acid analysis was performed using an LKB Model 4400 Amino Acid Analyzer according to the standard methods for determination of free amino acids in blood plasma, as described by Bruinvels and Pepplinkhuizen (1984).

Twenty-four hours after AIA administration rats were injected intraperitoneally with 2 mmol/kg serine (Merck, biochemical grade) dissolved in 0.5 ml saline, or with the vehicle solely. Thirty minutes after serine injection, catalepsy was measured by placing the rat on a vertical grid with a grid size of 1.5x1 cm, about 10 cm above the cage floor. The time the animal did not displace one of its front- or hind paws was recorded. This measurement was repeated twice and the mean duration was calculated. Immediately after the three catalepsy measurements 2 blood samples (0.4 ml and 10 ml for plasma amino acid and for beta-carboline determination, respectively) were collected by heart puncture under light ether anaesthesia, and beta-carbolines were determined in platelet rich plasma according to the method described by Schouten and Bruinvels (1985).

RESULTS

The induction of porphyria was checked by measuring delta-aminolevulinic acid (delta-ALA) and porphobilinogen (PBG) excreted into 24 hr urine (Table 1). A significant increase in delta-ALA excretion, and a massive increase in PBG excretion was measured in the AIA pretreated rats, indicating that these rats had developed a severe porphyria.

	pretreatment			
porphyrin	Controls	AIA	% Change	2P<
delta-ALA	490 ± 32	898 ± 118	+ 83.2 %	0.005
PBG	24.5 ± 3.5	753 ± 115	+ 2970 %	0.001

Table 1. delta-Aminolevulinic acid (delta-ALA) and porphobilinogen (PBG) excretion (nmoles/24 hr ± SEM) into urine during 24 hr after s.c. injection of 400 mg/kg allylisopropylacetamide (AIA). Statistical calculations were performed using Students' t-test; N=42).

Determination of plasma amino acids 24 hrs after AIA-pretreatment revealed a significant decrease of about half of the plasma amino acids (Table 2) The greatest decrease was found for glycine (43,9 %), while arginine, tyrosine and serine were decreased for about 40 percent. No significant differences in plasma concentration were measured for the amino acids not mentioned in Table 2.

The norharman concentration in platelet rich plasma (PRP) was signficantly increased after treatment of rats with AIA or serine. Although AIA + serine treatment caused a further increase of NH concentration, the latter was not significantly different from controls due to the large standard error. The concentrations of 6-hydroxytetrahydro-beta-carboline (6-OH-THBC), 6-methoxytetrahydro-beta-carboline (6-MeO-THBC), tetrahydro-beta-carboline (THBC) and 1-methyl-beta-carboline (harman, 1-Me-BC) in PRP were below limits of detection. Catalepsy was significantly increased after treatment with AIA + serine, as compared to both control and AIA treated rats, and after serine treatment when compared to control rats. However, rats treated solely with AIA did not show any cataleptic behaviour. During the cataleptic episode, catatonic phenomena like Straub tail, were also observed (data not

shown).

amino acid	pretreatment: Controls	pretreatment: AIA	% Change	2P<
glycine	451 ±16	253 ±14	- 43.9 %	0.0001
arginine	134 ± 3	80.0 ± 4.2	- 40.3 %	0.0001
tyrosine	95.8 ± 2.5	57.5 ± 1.8	- 40.0 %	0.0001
serine	293 ±11	178 ± 4.4	- 39.2 %	0.0001
glutamic acid	86.7 ± 5.3	53.4 ± 6.7	- 38.4 %	0.0005
asparagic acid	12.3 ± 1.0	7.9 ± 0.4	- 35.4 %	0.0005
ornithine	55.6 ± 3.1	38.0 ± 2.7	- 31.6 %	0.0001
citrulline	50.5 ± 1.3	34.9 ± 1.7	- 30.9 %	0.0001
alpha-ABA	14.4 ± 1.0	10.3 ± 1.0	- 28.5 %	0.01
alanine	266 ± 9	194 ±11	- 27.1 %	0.0001
threonine	212 ± 6	156 ± 5	- 26.4 %	0.0001
taurine	145.3 ± 7.2	110.7 ± 5.7	- 23.8 %	0.0001
methionine	49.9 ± 0.9	42.9 ± 2.1	- 14.0 %	0.002
histidine	55.9 ± 1.4	49.2 ± 2.0	- 11.7 %	0.01

Table 2. Plasma amino acids (mM ± SEM) in starved porphyric rats, measured 24 hrs after AIA treatment, just before serine injections. Statistical calculations were performed using Students' paired t-test (N=18).

treatment	catalepsy	norharman	serine	glycine
Control	3.7 ± 2.1*	0.57 ± 0.03	267 ± 20	453 ± 32
AIA	4.1 ± 1.2	1.08 ± 0.19+	205 ± 20+	300 ± 30++
Serine	13.3 ± 4.4+	1.63 ± 0.34+	778 ± 61+++	409 ± 31
AIA + Serine	24.9 ± 4.2+++,#	1.88 ± 0.85	663 ± 38+++	319 ± 30++

Table 3. Catalepsy duration (sec), NH concentration in platelet rich plasma (nM) and serine and glycine plasma concentration (uM) in porphyric rats 30 minutes after 2 mmol/kg serine i.p. injections. Statistical calculations were performed using the Mann-Whitney-U-test for the catalepsy data and Students' t-test for the other parameters.
* data represent mean ± SEM (N=9).
\+ P<0.05, ++ P<0.005 and +++ P<0.002 vs. controls.
\# P<0.005 vs. AIA.

After serine injections, serine plasma levels were 3-fold increased when compared to controls and AIA pretreated animals (Table 3). Glycine plasma levels were decreased in AIA pretreated animals, and did not change significantly after serine administration.

When the duration of catalepsy was plotted against the norharman concentration in PRP, these data showed best fitting into an exponential curve (Fig. 1). No correlation was found between delta-ALA or PBG excretion and norharman PRP-levels during porphyria (data not shown).

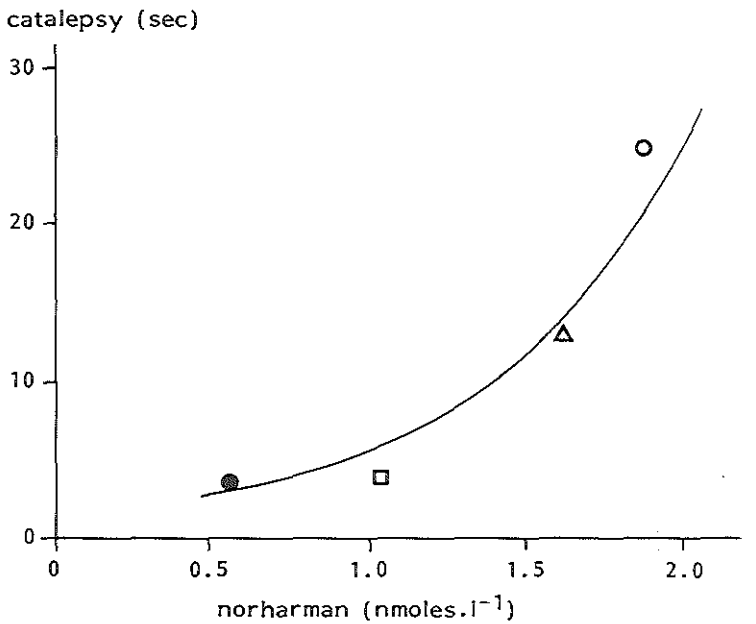

Fig. 1. Correlation of norharman concentration in PRP (nmoles/l) vs. catalepsy duration (sec). Each value represents the mean of 9 observation pairs. Curve fitting on the data in table 3 indicated that an exponential fit showed best correlation (R=0.894).
Legend to figure: ● =control;
□ =AIA;
△ =serine;
○ =AIA + serine.

DISCUSSION

Many suggestions have been put forward concerning the endogenous formation of beta-carbolines and its relation to a number of behavioural disturbances (Bruinvels et al., 1980; Honecker et al., 1980; Kari et al., 1979; Rimon et al., 1984). Previously, an animal model for an acute psychosis has been developed, using administration of serine to starved porphyric rats assuming that beta-carbolines could be formed endogenously under these circumstances (Schouten and Bruinvels, 1983). The present experiments were designed in order to show the presence and to measure the concentration of 5 different beta-carbolines in serine treated porphyric rats using a chromatographic method where the artifactual formation of beta-carbolines was excluded or at least below the limit of detection (Schouten and Bruinvels, 1985).

In the present study, a severe porphyria developed after AIA administration to starved rats, resembling acute intermittant porphyria (AIP) as indicated by the massive increase of delta-aminolevulinic acid (delta-ALA) and porphobilinogen (PBG) excretion into urine (Table 1). It has been shown that AIA depletes the heme pool by inducing cytochrome P 450 activity, thus causing strong induction of the enzyme delta-aminolevulinate synthase (delta-ALAS), the first enzyme in the heme pathway, in liver (Fig. 2; Marver et al., 1966; Unseld and de Matteis, 1978). As a result, the demand for glycine will be increased in these porphyric animals. Indeed it has been shown that plasma glycine levels were decreased in porphyric rats, and a negative correlation was found between delta-ALA excretion and glycine plasma levels (Schouten and Bruinvels, 1983). The present results show that plasma levels of serine and threonine were also significantly lowered during AIA-porphyria (Table 2), probably because the increased amount of glycine needed for porphyrin biosynthesis is produced from these amino acids by serine hydroxymethyltransferase (SHMT). Since the K_m values of SHMT for serine and glycine are low, the enzyme will be nearly saturated with its substrates in vivo. The direction of the equilibrium reaction will therefore be determined by the intracellular concentrations of each of the substrates (Snell, 1984). In porphyria, where the demand for glycine is increased, glycine concentrations were significantly lowered and thus more serine must be converted into glycine (see Fig. 2). However, in the present experiments serine administration did not increase glycine plasma levels, and therefore does not favour an increased conversion of serine into glycine in both porphyric and non-porphyric animals (Table 3). An explanation for this failure may be that metabolic pathways which convert glycine

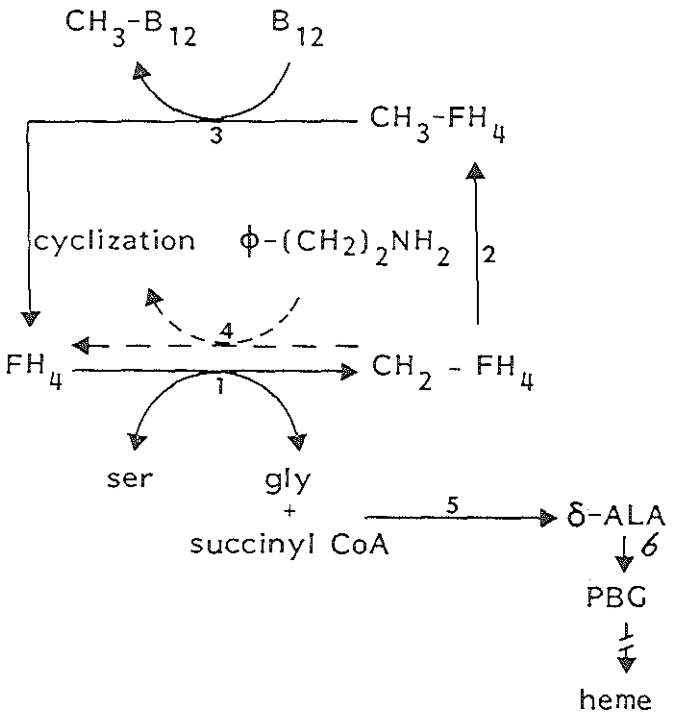

Fig. 2. Metabolic pathways of the one-carbon cycle, serine- and glycine metabolism and the heme pathway.
1. Serine hydroxymethyltransferase (SHMT).
2. Methylenetetrahydrofolate reductase (CH_2-FH_4).
3. Methyltetrahydrofolate: homocysteine methyltransferase.
4. Non-enzymatic cleavage of CH_2-FH_4 to FH_4 and formaldehyde, followed by the Pictet-Spengler reaction.
5. delta-Aminolevulinic acid synthetase (delta-ALAS)
6. Porphobilinogen synthase (PBGS)
ϕ-$(CH_2)_2NH_2$ = indoleamine or catecholamine

into other products occur too fast in the rat to measure an increased formation of glycine in plasma 30 min after serine administration in contrast to results obtained in man (Bruinvels and Pepplinkhuizen, 1984).

The expected increased conversion of serine into glycine will simultaneously increase the production of CH_2-FH_4 from tetrahydrofolate (FH_4, Fig. 2; Bruinvels et al., 1980). Studies of Pearson and Turner (1979) showed that THBC's can be synthesized

in vitro under physiological conditions from tryptamine in the presence of serine and FH_4 using partially purified SHMT. Methylenetetrahydrofolate, which is formed by this reaction, can non-enzymatically decompose into FH_4 and formaldehyde (HCHO). The latter substance can react with indoleamines forming THBC's via the Pictet-Spengler reaction, probably because other enzymes of the one-carbon cycle are not present in this preparation. Nevertheless, an increased conversion of serine into glycine in vivo may also result in the formation of beta-carbolines (McIsaac, 1961). The present results indeed show that norharman (NH) is formed in porphyric rats after serine injection, but instead of norharman one would rather expect the formation of THBC or of 6-hydroxy-THBC from the physiological substances tryptamine and 5-hydroxytryptamine respectively. An explanation might be that the concentration in plasma of hydrogenated precursors of NH do not reflect concentrations in tissues where these compounds are formed. One may therefore assume that dehydrogenation of the formed THBC, which is known to occur in rats (Greiner and Rommelspacher, 1983), accounts for the formation of NH.

There has been some discussion whether determination of THBC's in biological samples using extraction procedures are reliable methods to quantitate these compounds (Bosin et al., 1983). Careful studies using C-18 reverse-phase sample clean-up cartridges in order to minimize the use of organic solvents, indicate that these compounds can be determined without any measurable artifactual formation of tetrahydro-beta-carbolines (Schouten and Bruinvels, 1985). In addition, artifactual dehydrogenation of THBC's in biological samples has, to our knowledge, not been reported to occur during work up procedures.

The present results show a positive correlation between catalepsy and plasma NH concentration as determined immediately after measurement of catalepsy. However, a relatively small increase of plasma NH resulted in a large behavioural response. Therefore, the question arises whether the plasma concentration of NH is causally related to the behavioural phenomena observed. It is not inconceivable that NH, which is a very lipophylic substance, easily enters the brain resulting in higher concentrations of NH in brain as compared to plasma. However, determination of NH in brains of porphyric rats have to be performed in order to support this suggestion. In addition, other substances like tetrahydroisoquinolines (THIQs) may be formed under the same conditions from catecholamines and these may also contribute to the cataleptic behaviour observed (Melchior and Collins, 1982). Nevertheless, some behavioural effects of NH resembling the behaviour observed in the present study, like a stiff tail, have been

found by Morin et al. (1981, 1984) after i.p. injections of 20 or 50 mg/kg NH in rats. Using the higher dose, they observed loss of righting reflex and a catatonic appearance with stiff and extended front and hind limbs. The authors also measured the in vivo concentrations of NH in rat brain cortex, which ranged from 4-16 uM after i.p. administration of 50-200 mg/kg NH respectively. These results suggest a causal relationship between the increased plasma concentration of NH and the cataleptic behaviour observed in the present study. However, the brain concentrations reported by Morin (1984) are much higher than the plasma concentration of endogenously formed NH in our animal model.

In conclusion, the present results show that in an animal model for acute psychosis using serine treated porphyric rats, a correlation was found between NH levels in PRP and the duration of catalepsy. In addition these results support the hypothesis that endogenous "psychotic" substances can be formed as a result of metabolic disturbances in serine and glycine metabolism. This metabolic disturbance and the endogenous formation of beta-carbolines are held responsible for the psychedelic symptoms in episodic psychotic patients where the psychedelic symptoms could be re-induced in recovered patients by oral administration of serine (Bruinvels et al., 1980; Pepplinkhuizen et al., 1980).

ACKNOWLEDGEMENTS

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CHAPTER V

OCCURRENCE OF THE beta-CARBOLINE NORHARMAN IN PLASMA OF PATIENTS DURING EPISODES OF ACUTE PSYCHOSIS.

by

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Udo A.Th. Brinkman and Lolke Pepplinkhuizen.

Summary

Beta-carbolines have been postulated to be endogenously formed in patients suffering from an acute psychosis, accompanied by psychedelic symptoms. These patients were diagnosed according to criteria described elsewhere. Blood samples were collected from 6 patients during an acute psychotic state, as well as from 3 patients with the same diagnosis, but in a psychotic free period, and from 8 healthy controls. Norharman (beta-carboline, BC), harman (1-methyl-BC), 1,2,3,4-tetrahydro-beta-carboline (THBC), 6-hydroxy-THBC (6-OH-THBC), and 6-methoxy-THBC (6-MeO-THBC) were extracted and analyzed using high-preformance liquid chromatography (HPLC) with fluorometric detection. A significant increase in norharman was found in platelet poor plasma (PPP) from acute psychotic patients during a psychotic episode, when compared with both patients in a symptom-free period, and healthy controls. These differences were not found in platelets. No changes of harman, THBC, 6-OH-THBC or 6-MeO-THBC concentrations in either platelets or PPP were found. The identity of norharman was confirmed using a newly developed liquid chromatography - mass spectrometry (LC-MS) method.

Chapter V

INTRODUCTION

Since the proposal by Osmond and Smythies (1952) that an abberant methylation of catecholamines might be the cause of schizophrenic psychoses, the search for psychotomimetic substances in biological fluids of patients over the past years was without success. Although dimethyltryptamine (DMT), a hallucinogenic derivative of tryptamine, was detected in cerebrospinal fluid of schizophrenic patients (Smythies et al., 1979), no difference between patients and controls was noted. Moreover, a much higher concentration of DMT was found in non-psychotic patients with liver disease.

To test the possibility that a disturbed transmethylation of catecholamines or of indoleamines can evoke acute psychotic symptoms, Pollin et al. (1961) administered large doses of methionine, the precursor of the methyldonor S-adenosylmethionine (SAM), to chronic schizophrenic patients for several days. It was reported that 40% of the treated patients showed some acute psychotic reaction. This investigation was followed by nine other studies none of which, however, could convincingly show that administration of methionine to chronic schizophrenics was responsible for schizophrenic symptoms (Cohen et al., 1974). Some years later Levi and Waxman (1975) argued that schizophrenic patients should have a reduced level of SAM. This was confirmed by Carl et al. (1978), who found that the activity of two enzymes involved in the biosynthesis of SAM, methionine adenosyltransferase (Fig. 1, step 3) and serine hydroxymethyltransferase (Fig. 1, step 1), was significantly lower in erythrocytes of schizophrenics as compared to a control group. One may therefore conclude that an increased biosynthesis of SAM, concurring with the transmethylation hypothesis, is unlikely. Smythies (1984) recently re-evaluated the transmethylation hypothesis and concluded that schizophrenics who respond to methionine with acute psychotic symptoms are more likely to have an under- than an overactive methylating system (Fig. 1, step 4).

It was previously reported (Pepplinkhuizen et al., 1980; Bruinvels and Pepplinkhuizen, 1984 and 1985) that a group of patients suffering from an episodic psychosis with psychedelic symptoms (dysperceptions) may be an example of a group of patients where endogenous formation of hallucinogenic beta-carbolines occurs (Pepplinkhuizen et al., 1980). Oral administration of serine (2 mmol/kg) to these patients, who had adequately recovered from acute psychotic attacks, caused a recurrence of psychotic symptoms within a few hours. During remissions, these

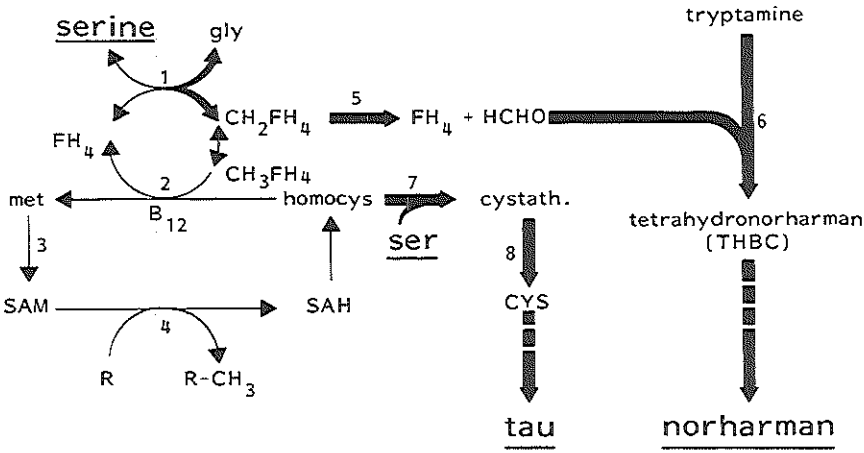

Fig. 1. Flow diagram showing serine metabolism in relation to taurine and norharman formation in acute psychotic patients. Substances underlined were shown to be altered, while thick lines indicate putative preferential pathways in this group of patients.

gly = glycine	SAM = S-adenosylmethionine
ser = serine	SAH = S-adenosylhomocysteine
met = methionine	FH_4 = tetrahydrofolic acid
homocys = homocysteine	CH_2=FH_4 = 5,10-methylene-FH_4
cystath. = cystathionine	CH_3-FH_4 = 5-methyl-FH_4
cys = cysteine	B_{12} = vitamin B_{12}
tau = taurine	HCHO = formaldehyde

patients showed a lower fasting plasma serine level, but a higher plasma taurine level (Bruinvels and Pepplinkhuizen, 1984). The lower serine level is explained by the enhanced conversion into glycine (Fig. 1, step 1) and by the increased formation of taurine via the condensation of serine with homocysteine forming cystathionine (Fig. 1, step 7). Recently, evidence was obtained for a factor present in the serum of these patients, responsible for the metabolic shift of homocysteine from the remethylation pathway (Fig. 1, step 2) to the transsulfuration pathway (Fig. 1, via steps 7 and 8; Fekkes et al, in preparation). As a consequence of the increased formation of taurine from serine, less homocysteine will be available for the remethylation to methionine (Fig. 1, step 2). This metabolic shift results in higher levels of CH_3-FH_4 and CH_2=FH_4 (Mudd and Poole, 1975), thus facilitating the dissociation of CH_2=FH_4 into FH_4 and formaldehyde (Fig. 1, step 5). The latter substance reacts non-enzymatically with indoleamines to form tetrahydro-beta-carbolines via the

Pictet-Spengler reaction (Fig. 1, step 6).

Since the finding of an enhanced conversion of serine into glycine and the increased formation of taurine indirectly support the proposed hypothesis, it was considered important to obtain direct evidence by measuring beta-carbolines in the plasma and platelets of acute psychotic patients. Therefore, a method developed recently (Schouten and Bruinvels, 1985) based on extraction of beta-carbolines from plasma and platelets, followed by high performance liquid chromatography with fluorometric detection, was used to measure tetrahydro-beta-carbolines and beta-carbolines in platelet poor plasma (PPP) and in platelets of acute psychotic patients. Moreover, the identity of a compound found in acute psychotic patients was confirmed using a newly developed liquid chromatography/ mass spectrometry (LC-MS) technique after derivatization of the compound found.

METHODS

Subjects

Blood was obtained from 8 healthy subjects with a mean age of 27 year (range 17-45), and from 9 patients with episodic psychosis, as diagnozed according to criteria described elsewhere (Pepplinkhuizen, 1983), of which 6 had an acute psychotic episode, and 3 were in a symptom-free period.

Materials

Norharman (BC) was obtained from Fluka. PFB.CL was obtained from Ventron, Karlsruhe, GFR. The organic solvents were obtained from Merck and were of Uvasol quality. 1,2,3,4-Tetrahydro-beta-carboline (THBC), 6-hydroxy-THBC (6-OH-THBC) and 6-methoxy-THBC (6-MeO-THBC) were synthesized according to the method described by Vejdelek et al. (1961). Reverse phase Sep-pak C-18 cartridges were obtained from Waters Ass.. Zorbax BP TM C8 was purchased from Chrompack (The Netherlands). Demineralized distilled water (demidest) was used as a solvent.

HPLC-fluorometry

Sample treatment. Venous blood (30 ml samples) was collected in 10 ml Vacutainer Thrombotect tubes containing 2.5% EDTA, 0.025% 2-chloroadenosine and 7.0% procaine-HCl, to inhibit platelet aggregation. These samples were separated into platelet poor plasma (PPP, 15 ml) and platelets (3.7×10^9 platelets), and stored at -80 ^{o}C until extraction. Beta-carbolines were extracted into a final volume of 1 ml extraction buffer, according to the method described previously (Schouten and Bruinvels, 1985).

Chromatography. High performance liquid chromatography was performed on a HP 1084 B Liquid Chromatograph (Hewlett Packard) using a stainless steel column (250 x 4.6 mm) home-packed with Zorbax BP TM C8 (7-8 micrometer; Chrompack). A Chrompack 75 x 2.1 mm Guard Column was used to protect the analytical column. The injection volume was 0.2 ml, while buffer flow was kept at 2 ml/min at a temperature of 45^{o}C. Chromatography was performed with a single buffer system containing 25 mM $(NH_4)_2HPO_4$ and 30 % 2-propanol in demidest. The pH was adjusted to pH 6.0. The compounds were detected by using an Aminco SPF 500 ratio fluorometer fitted with a 35 microliter flow cell. The fluorescence was measured using an excitation wavelength of 375 nm (bandwith 15 nm) and an emission wavelength of 434 nm (bandwith 40 nm). Quan-

titation was performed by automatic peak area integration using the Hewlett Packard 79850B terminal, as described earlier (Schouten and Bruinvels, 1985).

Liquid chromatography - mass spectrometry (LC-MS)

Extraction. After chromatography using the method described above, the fraction of the HPLC-eluate containing the norharman peak (3 ml = approx. 1.5 min) was collected and extracted at pH 9.0 with 2 x 1 ml ethyl acetate. The organic phase was evaporated until dryness, and the residue was derivatized and reanalyzed using the HPLC-MS technique described below.

Derivatization. Derivatization of norharman was performed in 100 microliter ethyl acetate containing 800 microM PFB.Cl and 1 mM trimethylamine, and was incubated for 30 minutes at 20°C. Excess PFB.Cl and other relatively volatile compound were evaporated under vacuum (2 mm Hg) until dryness.

LC. The mobile phase was delivered using a Gilson (Villiers-le-Bel, France) Model 320 pump. Samples were introduced using a micro injection valve with a 0.5 microliter internal injection loop. The stainless steel column (100 x 1.1 mm) was packed with RP-6 fully capped Phase Sep reverse phase stationary phase, and eluted with acetonitrile, at a flow rate of 25 microliter/min. This column was connected on-line with the mass-spectrometer via a DLI interface.

MS. A Finnigan (Sunnyvale, CA, U.S.A.) Model 4021 quadrupole mass spectrometer was used, while a Finnegan Model 2100 INCOS data system was employed for data acquisition and processing. The MS ion-source temperature was 300°C. The direct liquid introduction (DLI) interface consists of a 35-cm long stainless-steel jacket which fits into the solid sample probe of the MS instrument. The LC effluent enters the MS source through a fused silica capillary (length 500 mm; 0.16 mm O.D. x 0.05 mm I.D.). This capillary was fitted coaxial with a stainless-steel capillary (1/16 in. O.D. x 0.3 mm I.D.). Helium (at an inlet pressure of 1-2 bar) flowed in between both capillaries into the MS source. Technical details of the DLI interface are given elsewhere (Apffel et al., 1983; Maris et al., 1985). The mass spectrum was recorded in the negative chemical ionization mode, using the HPLC effluent (acetonitrile) as a reaction gas.

RESULTS

For HPLC analysis with fluorometric detection 30 ml blood samples were drawn from episodic psychotic patients during an acute psychotic episode, from patients with the same diagnosis but in a psychotic-free period and from healthy controls. As can be seen from Fig. 2, a compound which co-elutes with norharman is found in PPP obtained from an acute psychotic patient, while much lower amounts were found in samples, obtained from a recovered psychotic patient and from a healthy control. In some cases small amounts of harman could be detected in PPP just above the detection limit (0.1 picomole/ml). However, these amounts were not significantly different between the three groups. PPP obtained

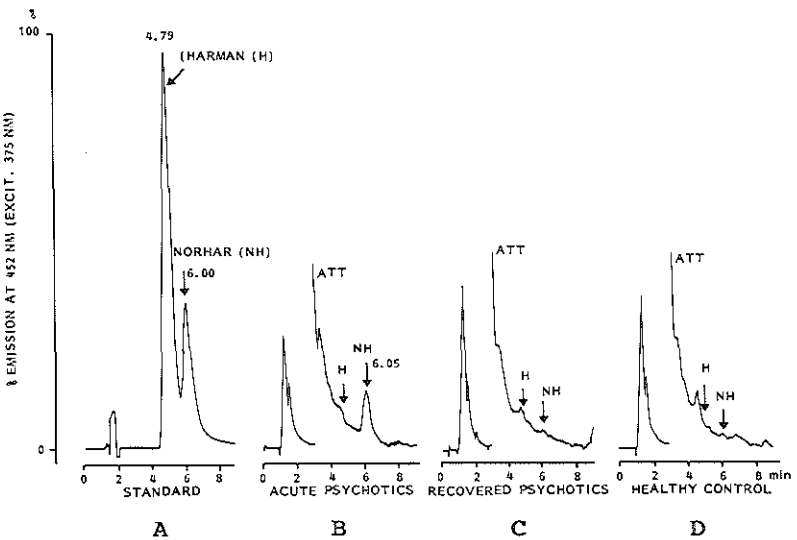

Fig. 2. HPLC chromatograms of patient platelet poor plasma (PPP) samples using fluorometric detection (see Methods). **A:** Standard sample, 200 pmol/compound; **B:** sample of an acute psychotic patient; **C:** Sample of a recovered patient; **D:** Sample of a healthy control subject. Norhar=norharmane; ATT=change of attenuation of emission signal.

from acute psychotic patients contained 0.54 ± 0.1 picomol (mean ± SEM) norharman per ml while norharman in PPP from healthy controls and symptome-free patients were below limits of detection (0.17 ± 0.03 picomol/ml and 0.11 ± 0.02 picomol/ml, respec-

tively; Fig. 3). In platelets from all groups, harman and norharman were below limit of detection. Also, there were no significant differences in the levels of THBC, 6-OH-THBC or 6-MeO-THBC, extracted from both PPP and platelets between the three groups studied (Schouten and Bruinvels, 1985).

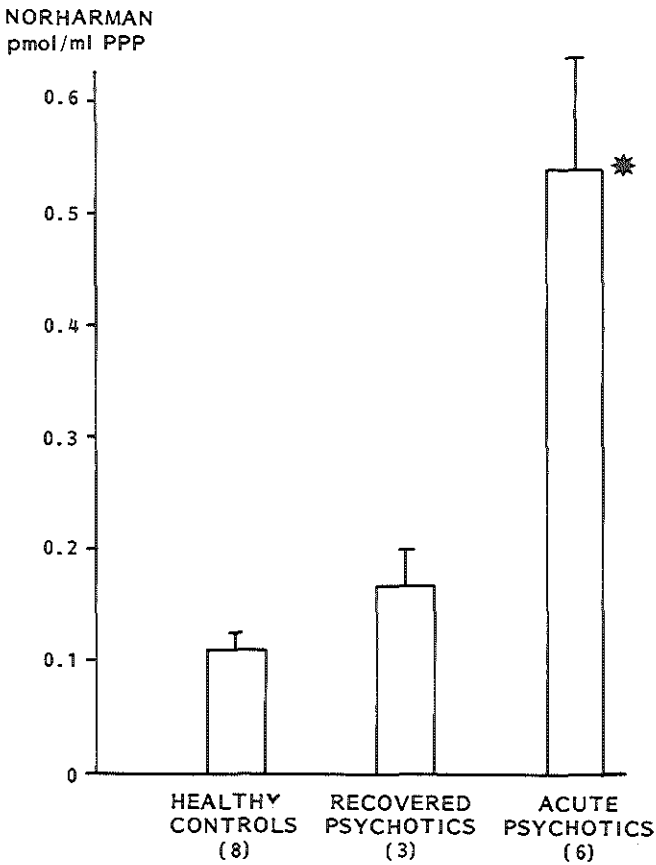

Fig. 3. Norharman (nM ± SEM) in platelet poor plasma (PPP) of acute psychotic patients (N=6), recovered psychotic patients (N=3) and healthy controls (N=8).
* P<0.005, Student's t-test.

The presence of a substance in plasma from acute psychotic patients which was chromatographically and fluorometrically not distinguishable from the beta-carboline norharman, was further

substantiated by LC-MS. Norharman could be derivatized easily using pentafluorobenzoyl chloride (PFB.Cl) which reacts with the indole nitrogen of norharman yielding a norharman-PFB derivative with a molecular weight of 362 (Fig. 4). This compound is very stable and could be chromatographed on a RP-6 column using acetonitril as the eluent. The column effluent was introduced on line into the ion source of the mass spectrometer and thus served as a reaction gas for negative chemical ionization. Under these conditions, approx. 25% of the ion is not fragmented (m/e 362), while 75% is present as a 342 fragment, probably due to splitting off of a hydrogenfluoride (HF) fragment (Fig. 4). This technique yielded a detection limit of 10 fmoles norharman-PFB using the 342 fragment for quantification in the single ion mode.

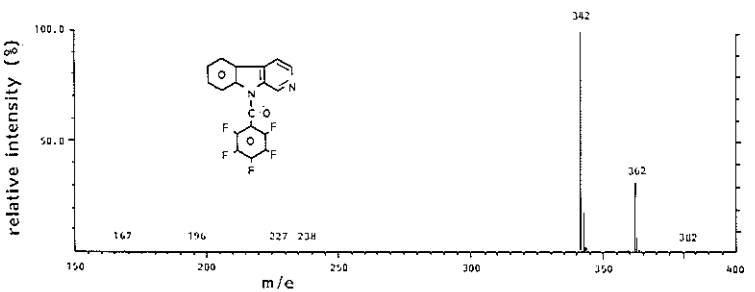

Fig. 4. Mass spectrogram of the norharman-pentafluorobenzoyl (NH-PFB) derivative (MW 362). 10 nmoles of norharman were derivatized using the method described in this report. The residue containing the NH-PFB derivative was dissolved in 20 microliter acetonitrile of which 0.5 microliter, representing 250 picomole of NH-PFB, was introduced into the LC-MS. Relative intensity was recorded in the multiple ion detection mode (MID), using the acetonitrile effluent as the reaction gas for negative chemical ionization (NCI).

In order to confirm the identity of norharman, as indicated in Fig. 2, two eluate fractions, corresponding with the norharman peak from either the norharman standard or from a PPP extract from a psychotic patient blood sample were extracted, and derivatized with PFB.Cl using the method described above. Fig. 5 shows two LC-MS chromatograms measured at m/e 342, using the single ion mode (SID). The main peak at m/e 342 of the patient sample had the same retention time as the norharman-PFB standard, establishing that the peak measured using the HPLC-fluorometry method indeed represents norharman. The two other peaks in the LC-MS chromatogram could not be identified using MID, due to the low

concentrations. However, corresponding peaks appeared in the LC-MS chromatograms of PPP extracts, obtained from healthy controls (chromatograms not shown).

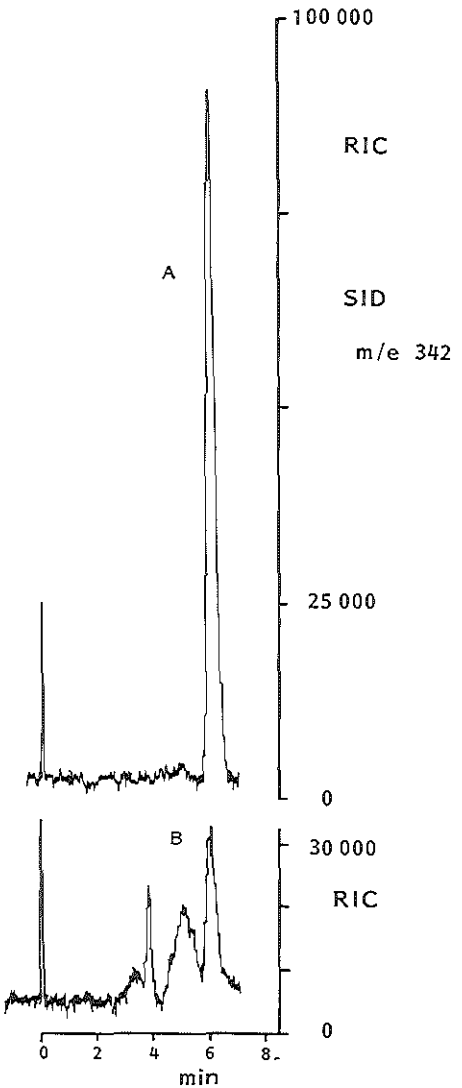

Fig. 5. HPLC-MS chromatogram of norharman-PFB. The reconstructed ion current (RIC) was recorded in the single ion mode (SID), using an m/e of 342. Both samples were extracted from the corresponding HPLC-fluorometry eluate and derivatized as described in the Methods. Residues were dissolved in 2 microliter acetonitrile, of which 0.5 microliter was introduced in the LC-MS.
A. Standard NH-PFB
B. PPP extract from an acute psychotic patient.

DISCUSSION

The present results indicate that platelet poor plasma of acute psychotic patients, experiencing psychedelic symptoms like distorted sensory perceptions (Pepplinkhuizen, 1983), contains the beta-carboline norharman. Maximum care was exercised to eliminate the possibility of artifactual formation of beta-carbolines (Schouten and Bruinvels, 1985). The presence of this compound was confirmed by LC/MS after derivatization. These results strengthen the original hypothesis that beta-carbolines are formed in these patients due to an enhanced serine metabolism (Bruinvels et al., 1980, Bruinvels and Pepplinkhuizen, 1985). The fact that norharman can be detected in plasma of patients recovered from their psychotic attacks or of healthy controls suggests that this substance may be responsible for one or more of the symptoms occurring during acute psychosis. However, as norharman concentrations, detected in PPP of these patients, are relatively low, it has still to be ascertained whether this compound is indeed responsible for one or more of these symptoms.

Norharman was also shown to be significantly increased in platelet rich plasma (PRP) of serine-treated porphyric rats (Schouten and Bruinvels, 1986). In addition, an exponential, positive correlation was found between the duration of catalepsy and the norharman concentration. From these results it was concluded that an increased conversion of serine into glycine is probably responsible for the formation of norharman.

Norharman has several neuropharmacological effects. It is a very potent ligand of the benzodiazepine receptor, evoking reverse effects of benzodiazepines ("inverse agonist"; Morin, 1984). It has been suggested that beta-carbolines are capable of inducing "anxiety", as opposite to effects of most benzodiazepines (Skolnick et al., 1984b). Moreover, norharman inhibits monoamine oxidase activity and the reuptake of 5-HT in vitro (Airaksinen and Kari, 1981 II). As low platelet MAO-A activity is suggested as a biological marker in schizophrenia, these mechanisms could be responsible for one or more of the symptoms of the patients studied. Further experiments are needed to obtain direct evidence for this assumption.

ACKNOWLEDGEMENTS

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CHAPTER VI

GENERAL DISCUSSION

Introduction

Many research groups have been putting effort in finding biochemical aberrations related to the etiology of psychoses, as discussed in Chapter I (Gillin, 1978; Smythies, 1982; Bruinvels and Peppinkhuizen, 1984). It has been suggested that excessive activity of methylation processes could result in the formation of abnormally methylated biogenic amines with hallucinogenic or even "psychotogenic" properties (Osmond and Smythies, 1952). Until now, these compounds have not been found in significant amounts, although some enzymatic abnormalities are present in different subgroups (Chapter I). In 1980 Pepplinkhuizen et al. published on a group of acute psychotic patients of the manic-psychedelic type, who showed a disturbance in serine- and glycine metabolism. These findings were used as a starting-point for this thesis. As described in Chapter I, an animal model was developed, based on an enhanced conversion of serine into glycine, in order to study the putative formation of beta-carbolines. The presence of these compounds was correlated with behavioural disturbances in these animals. Moreover, beta-carbolines were measured in plasma, prepared from blood from episodic psychotic patients. In the following paragraph, results from these studies are discussed.

Chemical porphyria in rats. Psychiatric manifestations are frequently present during acute intermittant porphyria (AIP). These symptoms may even occur during remission and become more prominent during an acute attack (Bowman and Rand, 1980). Goldberg and Stinnet (1983) reported of a porphyric patient with signs and symptoms of an acute psychotic and schizophrenic episode, resembling the symptoms as described by Peppinkhuizen et al., (1980). This patient was subsequently diagnozed as having AIP (Goldberg and Stinnet, 1983) . As described by Pepplinkhuizen (1983), 10% of a group of acute psychotic patients were also suffering from porphyria, probably AIP. It was argued , that an increased expenditure of glycine will be the result of acute porphyric attacks, as glycine is an ultimate precursor in the heme pathway (Chapter I. Therefore, the chemical induction of porphyria in rats was used as a method to increase the expenditure of glycine.

Two types of porphyrinogenic agents were used in the initial studies (Chapter II). After injection of lead acetate (25 mg/kg i.v.) in combination with phenobarbital (50 mg/kg i.p.), the excretion per 24 hr into urine of delta-aminolevulinic acid (d-ALA), the first product in the heme pathway, showed a 28-fold increase, whereas excretion of PBG, the second product in the heme pathway, was doubled. When administered simultaneously, lead and phenobarbital have a strong potentiating porphyrinogenic activity (Maxwell and Meyer, 1976). Lead is a potent inhibitor of enzymes containing sulfhydryl groups at the active site of the enzyme (Moore et al., 1980). Therefore, the main porphyrinogenic activity of lead is probably mediated by the interaction with PBG synthase and other enzymes containing sulfhydryl groups at their active sides (Moore et al., 1980). The massive increase of d-ALA, compared to a relatively low increase of PBG excretion into urine after lead + phenobarbital treatment can be explained by induction of d-ALA by phenobarbital, combined with increased breakdown of heme via the P450 system, and an inhibition of PBG synthase by lead (Maxwell and Meyer, 1976). The second method to induce porphyria consisted of subcutaneous injections of allylisopropylacetamide (AIA) into fasting rats. These rats excreted 4 times more d-ALA when compared to controls, while PBG excretion was increased 35-fold in these animals. It can be concluded from these data that both agents were strongly porphyrinogenic in rats, which is in agreement with other results (Maxwell and Meyer, 1976; Marver et al., 1966). However, the different profiles in d-ALA and PBG excretion indicate that these porphyrinogens act at different steps in the heme pathway. The porphyrinogenic action of AIA is mainly exerted by accellerating the breakdown of heme, thereby inducing the activity of d-ALA synthase

(Marver et al., 1966; Stephens et al., 1978; Unseld and De Matteis, 1978). As indicated by the massive increase of PBG excretion, excess d-ALA is mainly converted to PBG via PBG synthase after AIA pretreatment.

Glycine levels in porphyric rats. Due to the enhanced demand for glycine during chemical porphyria, glycine levels were negatively, semilogarithmically correlated with the excretion of d-ALA (Fig 3, Ch. II). It can be concluded from this observation that the conversion of serine via serine hydroxymethyltransferase (SHMT) will be increased, as serine is the main source for glycine (Arnstein, 1954). Therefore, it would be expected that serine plasma levels are also lowered during chemical porphyria. However, in AIA pretreated animals serine plasma levels were not changed, while in lead + phenobarbital pretreated rats these levels were increased (Fig. 2 Ch. II). As SHMT also contains sulfhydryl groups at its active site, it is probably inhibited by lead (Schirch et al., 1980). Because increased conversion of serine into glycine via SHMT is essential in the development of an animal model for this type of psychosis, it can be concluded from this point of view that AIA pretreatment of rats probably represents a more useful animal model for psychosis than pretreatment with lead + phenobarbital.

Serine and glycine administrations in porphyric rats. At maximum porphyria, which is 18 hrs after administration of lead + phenobarbital (Maxwell and Meyer, 1976) or 24 hrs after AIA administration (Marver et al., 1966), rats were injected with 2 mmol/kg serine or glycine. Serine plasma levels showed a twofold increase 20-30 minutes after serine injections in all pretreated rats, whereas an approx. 3-fold increase in glycine plasma levels was found 20 min after glycine injections (Chapter II). Serine plasma levels were increased only in AIA-pretreated rats 20-60 minutes after glycine injection, while glycine levels were increased 20 minutes after serine injection. These changes in serine and glycine plasma levels were not found in lead + phenobarbital pretreated rats, which can be explained by the inhibition of SHMT by lead, as described above. Moreover, serine- or glycine injections in non-pretreated animals did not result in increasing glycine- or serine plasma levels, respectively. These results indicate that only in AIA pretreated animals the interconversion of serine and glycine via SHMT is enhanced.

Catalepsy scores in porphyric rats after serine- or glycine administrations. The main problem which is encountered developing an animal model for psychotic behaviour in man is to find a useful parameter which can be used as an indicator for this type of

behaviour (Domino, 1976; Stoff et al., 1978; Smythies et al., 1969). In the group of patients under study, visual dysperceptions and hallucinations are prominent at the onset of the psychotic attack (Pepplinkhuizen, 1983). It could be argued then to develop an animal model in which hallucinogenic symptoms are measured. However, as reviewed by Domino (1976), there were no satisfactory animal models for studying hallucinogenic effects of indole compounds at the onset of these studies. Most of the existing methods were either nonspecific or very time consuming (Domino, 1976). Moreover, the duration of the cataleptic behaviour as measured in the porphyric rats did not allow the usage of these methods. Other types of behaviour which are frequently used as an indication of psychotic behaviour are catatonia and catalepsy (Bloom and Segal, 1972; Fisher, 1975; Maxwell, 1968; Smythies, 1969). These phenomena can be described as a rigid- and non-rigid type of immobility, respectively (Costall and Naylor, 1974). As many of the patients under study also suffered from catatonia at the height of their psychosis, measurement of this type of behaviour in rats could be suitable as an indicator for psychotic behaviour (Bruinvels et al., 1980). The vertical grid test was used in these studies for the measurement of catalepsy (Segal et al., 1977). Cataleptic behaviour was observed in both AIA and lead + phenobarbital pretreated animals after glycine injection, and only in AIA pretreated animals after serine injection. As explained earlier, lead could inhibit SHMT, which would prevent serine to be converted to glycine in excessive amounts. The finding that glycine can evoke catalepsy in porphyric rats, and psychotic effects in some of the psychotic patients (Bruinvels et al., 1980) appears to be somewhat contradictory to the hypothesis that enhanced conversion of serine into glycine will produce excessive amounts of CH_2=FH_4 (Chapter I). One would expect that SHMT should react in the opposite direction after glycine administration (Arnstein, 1954). However, glycine can be converted to CO_2 and NH_3 by the glycine cleavage system (GCS), also using FH_4 as a cofactor, which is converted into CH_2=FH_4. This appears to be a preferential metabolic pathway of glycine metabolism after a sudden increase in glycine concentration.

It can be concluded from the results discussed above that serine or glycine administrations to AIA-porphyric rats may probably represent a suitable model for studying an episodic, acute psychosis as described previously (Bruinvels et al., 1980; Pepplinkhuizen, 1983). As hypothesized in the Introductory part (Chapter I), beta-carbolines (THBCs or BCs) or tetrahydroisoquinolines (THIQs) could be formed as a result of excess formaldehyde production during the existence of high levels of CH_2=FH_4. Therefore, it will be most interesting to measure whether these

compounds are actually being formed during catalepsy in these porphyric rats.

Elimination of artifactual formation of beta-carbolines during work-up. Formaldehyde is present in many organic solvents and in most mammalian tissues and body fluids (Bosin et al., 1983). Biogenic amines like indoleamines or catecholamines can easily condense with formaldehyde via the Pictet-Spengler reaction forming tetrahydro-beta-carbolines (THBCs) and tetrahydroisoquinolines (THIQs), respectively (Chapter I). Therefore, procedures for extraction of beta-carbolines from mammalian tissues using substantial amounts of organic solvent could possibly introduce the artifactual formation of THBCs or THIQs, as studied by Bosin et al. (1983), and several other research groups (McKenna and Towers, 1984; Melchior and Collins, 1982; Airaksinen and Kari, 1981 I). In order to avoid these problems, a new method was developed in these studies using reversed-phase sample preparation cartridges for the extraction of beta-carbolines from plasma and platelets (Chapter III). Bosin et al. (1983) showed that artifactual formation still occurred during their alkalinization-extraction procedure. This could be eliminated by reducing the exposure of platelet or plasma samples to alkaline organic solvents. As beta-carbolines are more easily formed at alkaline pH (Whaley and Govindachari, 1951), the use of lower pH values during extraction (pH 4.0) will inhibit the Pictet-Spengler reaction (Hahn and Ludevig, 1934). However, addition of semicarbazide to extraction solvents as a formaldehyde trapping agent remained necessary. Using this method, artifactual formation of beta-carbolines was not detectable.

Beta-carbolines in plasma and platelets from man and rat. Beta-carbolines have been detected by many research groups in several tissues and body fluids in man and rat (Chapter I). Rommelspacher et al. (1979, 1982) reported that 6-hydroxy-1,2,3,4-tetrahydro-beta-carboline (6-OH-THBC) was present in rat platelets and plasma. As reported in Chapter III, these results were only confirmed for human platelets. Opposite to other groups, our method could not detect 6-methoxy-THBC (6-MeO-THBC) in human or rat plasma and platelets (Chapter I and III). As 5-methoxy-tryptamine (5-MeO-TA) reacts more readily with formaldehyde, forming 6-MeO-THBC, than 5-hydroxy-tryptamine (5-HT, serotonin) or tryptamine (TA, Bosin et al., 1983), data showing the presence of 6-MeO-THBC in mammals should be considered with great care (Bosin et al., 1983, Chapter III). These considerations are supported by the observation that concentrations of THBC and 6-OH-THBC found in these studies are in accordance with those found by other research groups (Chapter III), which also indicates that

TA and 5-HT are less prone to react with formaldehyde than 5-MeO-TA.

Low amounts of 1-methyl-beta-carboline (1-Me-BC, harman) were found in human platelets, which have also been detected in unknown quantities by Bidder et al. (1979). Harman is thought to be biosynthesized by the condensation of tryptamine and acetaldehyde (Stahl, 1977). Recent attempts to understand the pharmacological and toxicological actions of ethylalcohol have increasingly focused on the properties of acetaldehyde, its major metabolite. This action involves the condensation of acetaldehyde with catecholamines or indoleethylamines to form THIQs or THBCs, respectively. An intriguing effect of some of these compounds after intraventricular administration to rats was a marked and prolonged increase in voluntary ethanol ingestion (Myers and Oblinger, 1977; Deitrich and Erwin, 1980). In this way, THIQs and THBCs could stimulate their own biosynthesis.

As a conclusion, some questions related to the analytical procedures used to establish the identity of beta-carbolines in vivo still exist. Measurable artifactual formation of these compounds during extraction, using the method described in Chapter III, could be excluded. The concentrations of beta-carbolines found in plasma and platelets from man and rat are in accordance with, or lower than concentrations reported by others.

beta-Carboline concentrations in platelet rich plasma from serine-injected porphyric rats. Studies performed by Pearson and Turner (1979) showed that THBC can be formed in vitro under physiological conditions from tryptamine in the presence of serine and FH_4 using partially purified serine hydroxymethyltransferase (SHMT). This observation supports the hypothesis that beta-carbolines can be formed in vivo as a result of increased serine to glycine conversion. The present results showed that norharman (beta-carboline) is formed in vivo in porphyric rats after serine injection (Chapter IV table 3). No significant amounts of 6-OH-THBC, THBC, 6-MeO-THBC or 1-Me-BC were detected. As norharman is not fully saturated, it can not be formed directly by the Pictet-Spengler reaction. The formation of THBC or 6-OH-THBC from tryptamine or serotonin would be expected, but could not be found according to the present results. These compounds could be formed as intermediate products, and dehydrogenated quickly by an as yet unknown reaction. Although THBC can be metabolized to norharman in rats, among other products (Greiner and Rommelspacher, 1983), these compounds could not be measured using the present method (Chapter IV).

Correlation of norharman levels in PRP and duration of catalepsy. An exponential correlation was found between catalepsy duration and PRP norhaman levels (Chapter IV, fig. 1). The shape of the curve in the present study showed a striking similarity with the curve found by Pannier and Rommelspacher (1981), who measured catalepsy after i.p. administration of 1-40 mg/kg THBC to rats. These results indicate that the same type of effect could be observed in both studies. However, it can not be ascertained from these results whether the catalepsy as measured by Pannier and Rommelspacher, or measured in the present studies, was evoked by norharman or THBC, or by both. On the other hand, this finding indicates that these behavioural disturbances could possibly be evoked by norharman. This was confirmed by results of Morin *et al.* (1981, 1984), who described a rigid catatonic-like appearance after i.p. administration of norharman (20 mg/kg), which could be abolished by diazepam. Measurement of *in vivo* concentrations in these rats demonstrated that these biological effects occur at doses which, during *in vitro* experiments, occupy a large proportion of benzodiazepine receptors (Skolnick *et al.*, 1982; Morin *et al.*, 1981). As norharman also competitively inhibits specific binding of [^{3}H]-diazepam in mouse brain homogenates at low concentrations (Table 2), it was suggested by Morin *et al.* (1984) that this compound is probably a ligand of the benzodiazepine receptor, with effects opposite to those of diazepam. As summarized in Table 1 and 2, norharman has several *in vivo* and *in vitro* effects in mammals. However, the *in vitro* inhibition of monoamine oxidase-A in mouse brain (Buckholtz and Boggan, 1977) and the reuptake inhibition in rat brain synaptosomes (Komulainen *et al.*, 1980) occurred at a relatively low norharman concentrations. Therefore, a combination of these pharmacological actions could also contribute to the behavioural effects observed in the present study. As already suggested in Chapter II, probably a

effects	inhibitor	dose	author	
Kindled seizures in rats/ catatonia	diazepam .05 mg/kg	20 mg/kg 20 mg/kg	Morin Morin	1984 1984
Convulsions in rats	-	25 mg/kg	Rommelsp.	1981
5-fold increase in plasma corticosterone concentration.	-	210 um/kg	Meyer	1976

Table 1. *In vivo* effects of norharman in mammals.

mixture of catatonic and cataleptic behaviour was observed in serine treated porphyric rats. These different behavioural profiles could imply the participation of different neurotransmitter systems (Costall and Naylor, 1974).

effects		EC/IC_{50} (uM)	author	
Inhibition of acetylcholinesterase		100	Skup	1983
Inhibition of epinephrine binding at platelet alpha-2 adrenergic receptor		80	Given	1983
Inhibition of binding to benzodiazepine receptors	diazepam	1.62	Cain	1982
	,,	1.6	Morin	1981
	flunitrazepam	8	Rommelsp.	1981
Inhibition of reuptake in rat brain synaptosomes	5-HT	38	Komulainen	1980
	DA	64	,,	
	NA	110	,,	
Inhibition of uptake in human platelets	5-HT	57	Airaksinen	1980
	DA	10	,,	
Monoamine oxidase inhibition in mouse brain and liver		20	Buckholtz	1977

Table 2. In vitro effects of norharman in mammalian tissue.

Measurement of beta-carbolines in psychotic patients. Using the method as described in chapter III, beta-carbolines were measured in plasma obtained from acute psychotic patients, from patients with the same diagnosis but in a psychosis-free period, and from healthy controls. Norharman was detected only in plasma from psychotic patients at a concentration of 0.5 pmole/ml, while plasma levels of the other groups were below limit of detection (0.1-0.15 pmole/ml). As discussed for the serine-injected porphyric rats, THBC is expected to be an intermediate product in the biosynthesis of norharman. However, tetrahydro-beta-carbolines could neither be detected in these patients, nor in serine-injected porphyric rats using these methods. As mentioned earlier, Greiner and Rommelspacher (1983) described the metabolic fate of THBC after intravenous administration to rats. One of the urinary metabolites was norharman, indicating that processes that dehydrogenate THBC to norharman are active within the mammalian body.

A new method for LC-MS determination of norharman extracted from plasma extracts. As described in chapter I and III,there is some doubt on the excistence of beta-carbolines *in vivo*, due to a lack of specificity of the methods employed, and due to the failure to eliminate artifactual formation of these compounds during sample preparation. As the identification of norharman was based only on a HPLC separation method, combined with fluorometric detection, it was most important to confirm the presence of norharman in psychotic patients using mass spectrometry methods. As rather low concentrations of norharman were found, a sensitive mass spectrometry technigue technique had to be used for the detection of norharman, *eg.* negative chemical ionization (NCI). However, norharman can not be detected in the picomole range using this techinque (unpublished results). Moreover, chromatography of the alkaloid norharman on HPLC reversed phase materials, or on GC often resulted in tailing peaks (Sokolowski and Wahlund, 1980). Therefore, a derivatization technique has to be used which covalently binds norharman to a strong electron capturing molecule. As norharman has a pyridine ring, containing a tertiary amine, the secundary indole amine group had to be used for the derivatization reaction. However, the indole nitrogen of norharman is quite stable and cannot be derivatized with anhydride reagents like heptafluorobutyryl anhydride, pentafluoropropionyl anhydride or acetic anhydride (unpublished results), which are reagents used for derivatization of the piperidine nitrogen of THBCs (Honecker and Rommelspacher, 1978b; Faull *et al.*, 1982; Beck *et al.*, 1982b; Bosin and Holmstedt, 1982; Rommelspacher *et al.*, 1982). Pentafluorobenzoyl chloride is a compound combining high NCI sensitivity with high reactivity for primary and secundary amines. This compound reacted with the indole nitrogen under relatively mild conditions, as described in chapter V. This yielded a molecule with a molecular weight of 362, which could be chromatographed easily with GC-MS (unpublished results) and LC-MS, and detected using NCI in the femtomole range (Chapter V).

Norharman as a putative biochemical substrate responsible for psychosis. The present results indicate that norharman could be detected in significant amounts in plasma of acute psychotic patients experiencing psychedelic symptoms like distorted sensory perceptions. This observation supports the hypothesis that beta-carbolines in these patients could be formed as a result of enhanced serine to glycine metabolism. The concentrations of norharman in patients during psychosis are quite low, and it remains to be established whether these concentrations could play any role in the development of psychotic symptoms. It can be expected that norharman exerts its "psychotogenic" effects via the central nervous system. Plasma norharman levels could there-

fore be a bad indicator for its levels in the brain. However, norharman does cross the blood-brain barrier (Morin, 1984), and if it is not broken down rapidly (Greiner and Rommelspacher, 1982), plasma norharman concentrations could be a true reflection of those in brain.

The main question is by which mechanism in brain norharman exerts its effects in the patients described (Chapter I). Norharman has several *in vivo* and *in vitro* effects (Table 1 and 2). Catatonia in rats was observed after intraperitoneal injection of 20 mg/kg norharman (Morin, 1984). This could be prevented by the administration of 0.05 mg/kg diazepam, which indicates that the *in vivo* effects are mediated by binding to the benzodiazepine receptor. This hypothesis is supported by findings that norharman is a very potent ligand of the benzodiazepine receptor *in vitro* (Table 2). Morin (1984) suggested that the catatonic effects are mediated through activity at benzodiazepine related sites, thereby acting as an inverse agonist. Moreover, it has been suggested that beta-carbolines are capable of inducing "anxiety" in primates (Skolnick *et al.*, 1984a). As many of the patients described earlier (Pepplinkhuizen, 1983) also suffered from anxiety preceding and during their psychotic episodes, it is very tempting to speculate that these phenomena could be mediated through the pharmacological action of norharman on the benzodiazepine receptor. Also, relatively low amounts of norharman can inhibit monoamine oxidase-A activity and the reuptake of 5-HT *in vitro*. Both low platelet MAO activity and dysfunction of central 5-HT metabolism may contribute to the typical psychotic symptomatology. Others have suggested that the may be used as biological markers in schizophrenia (Chapter I), these pharmacological effects of norharman could also contribute to the phenomena observed in the patients studied.

The finding that this pharmacologically active compound is detected in episodic psychotic patients can be considered as a novelty in biological psychiatry, and could have implications for the diagnostic procedures employed to discriminate between different types of psychoses. These results may open new areas for fruitful research into the mechanism of action, the *in vivo* and *in vitro* formation, the concentrations in different parts of the mammalian brain during psychotic episodes in man and rat, and into the metabolism of this intriguing compound.

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ABBREVIATIONS

1-Me-BC = 1-methyl-beta-carboline
5-HT = 5-hydroxy-tryptamine
6-MeO-THBC= 6-methoxy-tetrahydro-beta-carboline
6-OH-THBC = 6-hydroxy-tetrahydro-beta-carboline
B_{12} = vitamine B_{12}
AIA = 2-allyl-2-isopropylacetamide
BC = beta-carboline
BCEE = beta-carboline-3-carboxylic acid ethyl ester
CH_2=FH_4 = methylenetetrahydrofolic acid
CH_3-FH_4 = methyltetrahydrofolic acid
CSF = cerebrospinal fluid
CYS = cysteine
CYST = cystathionine
d-ALA = delta-aminolevulinic acid
DMPEA = dimethoxyphenylethylamine
DMT = dimethyltryptamine
FH_4 = tetrahydrofolic acid
GC = gas chromatography
GLY = glycine
HCHO = formaldehyde
HCYS = homocysteine
HPLC = high performance liquid chromatography
LC = liquid chromatography
MAO = monoamine oxidase
MAT = methionine adenosyltransferase
MET = methionine
MS = mass spectrometry
OMB = O-methyl bufotenine
PBG = porphobilinogen
pmol = picomol
SAH = S-adenosyl homocysteine
SAM = S-adenosyl methionine
SEM = standard error of the mean
SER = serine
SHMT = serine hydroxymethyltransferase
Suc-CoA = succinyl-coenzyme A
TAU = taurine
THBC = tetrahydro-beta-carboline
THIQ = tetrahydroisoquinoline
ul = microliter
umol = micromol
V_{max} = maximal enzymatic conversion rate

SUMMARY

Many research groups have searched for putative biochemical substrates responsible for psychosis. As psychosis is apparently a heterogenous disease, several biochemical theories on the etiology of this disease coexist. These theories can be divided into those hypothesizing enzymatic aberrations as a possible cause for some types of psychosis, and those in which the formation of hallucinogenic or "psychotogenic" compounds is held responsible for the development of this disaese. In Chapter I, a summary is given of the current knowledge on this subject. The postulate that an increased conversion of serine into glycine, resulting in increased production of $CH_2=FH_4$, could give rise to the production of putative psychotogenic beta-carbolines, was used as a starting point in this thesis.

It was investigated in Chapter II whether an increased demand for glycine, as postulated to occur in patients who have suffered from episodic psychoses accompanied by multiple perceptual distortions, could evoke psychotic reactions. Catalepsy was used as a measure for psychosis, and was observed after injection of serine or glycine in porphyric rats. Catalepsy was shown to occur after serine as well as glycine administration in 2-allyl-2-isopropylacetamide (AIA) pretreated rats, while in lead + phenobarbital pretreated rats only glycine was effective. Administration of AIA to rats resulted in a strongly enhanced excretion of porphobilinogen (PBG) in urine, while lead + phenobarbital pretreated rats showed increased excretion of d-aminolevulinic acid (d-ALA). The lead + phenobarbital pretreated animals showed elevated serine plasma levels and lowered glycine plasma levels 18 hours after injection, while no significant differences in plasma levels of these amino acids were found 24 hours after AIA administration. In AIA or saline pretreated animals, but not in those pretreated with lead + phenobarbital, glycine formation from serine was elevated. It is concluded that the present animal model can be used to investigate episodic psychoses.

In Chapter III, the development of a fast method for extraction and concentration of tryptamine (TA), 5-hydroxy-TA and 5-methoxy-TA is described using reverse phase C-18 sample preparation columns in combination with an ion-pairing reagent. Using this method, 1,2,3,4-tetrahydro-beta-carboline (THBC), 6-hydroxy-THBC and 6-methoxy-THBC, the respective reaction products formed after reaction of formaldehyde with the primary amines mentioned above, and beta-carboline (BC, norharman) and 1-methyl-beta-carboline (1-Me-BC, harman) could be extracted from human and rat

platelets and platelet poor plasma (PPP). A HPLC method combined with fluorometric detection was developed for the quantitative determination of these compounds in the picomole range. The formation of beta-carbolines during the extraction procedure was below the limit of detection of the assay procedure. 6-OH-THBC, THBC, 1-Me-BC and 5-HT were identified as normal constituents of human platelets, whereas only 5-HT and 6-OH-THBC could be identified in human PPP. In rat platelets and PPP 5-HT, but no THBC's could be detected.

Porphyria was induced in adult male Wistar rats starved for 24 hrs by s.c. injection of 400 mg/kg allylisopropylacetamide (AIA), as described in Chapter IV. The presence of porphyria was shown by measuring excretion of delta-aminolevulinic acid (delta-ALA) and porphobilinogen (PBG) into the urine during 24 hr after AIA administration. Plasma levels of glycine, serine and of a number of other amino acids were decreased in porphyric rats as compared to controls. Intraperitoneal injection of 2 mmol/kg serine 24 hr after AIA administration was used as an animal model for an acute psychosis, by measuring catalepsy scores 30 min after serine injection. The concentration of 5 different beta-carbolines in platelet rich plasma (PRP) was measured using an HPLC-fluorometric method. An increase in the concentration of norharman (NH) in PRP, ranging from 0.57 nmoles/l in control rats to 1.88 nmoles/l in serine treated porphyric rats was found. The catalepsy duration was exponentially correlated with the NH concentrations in PRP. It is concluded that an elevated conversion of serine into glycine via serine hydroxymethyltransferase (SHMT) may be responsible for the enhanced NH biosynthesis.

In a group of patients having recovered from episodic psychotic attacks oral administration of serine precipitated psychotic symptoms within 2-4 hours supporting the hypothesis that an increased conversion of serine into glycine results in an accumulation of methylene tetrahydrofolic acid (CH_2=FH_4) in these patients (Pepplinkhuizen, 1983). It is well known that CH_2=FH_4can decompose non-enzymatically into tetrahydrofolic acid and formaldehyde (Chapter I). The latter substance can react with indoleamines or catecholamines via the Pictet-Spengler reaction forming tetrahydro-beta-carbolines and tetrahydroisoquinolines, respectively. It is reported in this chapter for the first time that the beta-carboline norharman is present in the plasma of acute psychotic patients. This substance was not detectable in plasma obtained from healthy control subjects or from symptom-free episodic psychotic patients. The occurrence of norharman in plasma may therefore represent an endogenously formed substance responsible for one or more of the psychotic symptoms of which one may

be anxiety.

It is concluded in Chapter VI that the occurrence of norharman, which is a pharmacologically very active compound, can be considered as a very important finding in biological psychiatry. These results were supported by the finding that the same compound can be detected in rats during behavioural disturbances after chemical enhancement of the conversion of serine into glycine, which probably also occurs in acute episodic psychotic patients.

SAMENVATTING

Veel onderzoeksgroepen over de hele wereld zijn al zo'n 30 jaar op zoek naar mogelijke biochemische substraten die verantwoordelijk zouden kunnen zijn voor psychotische aandoeningen. Daar deze ziekte een duidelijk heterogeen karakter draagt, bestaan er ook meerdere biochemische theorieen betreffende de oorzaak van psychosen. In grote lijn kunnen deze theorieen worden verdeelt in diegene welke een enzymatische afwijking postuleren, en diegene waarin de vorming van hallucinogene of "psychotogene" stoffen verantwoordelijk worden gesteld voor deze ziekte. In Hoofdstuk I wordt een samenvatting gegeven van de huidige kennis van zaken van enkele van deze theorieen. De aanname dat een toegenomen omzetting van serine in glycine, resulterend in toegenomen productie van methylene tetrahydrofoliumzuur (CH_2=FH_4), de productie van mogelijk psychotogene beta-carbolines tot gevolg zou kunnen hebben, werd gebruikt als uitgangspunt in dit proefschrift.

In hoofdstuk II is het onderzoek beschreven waarin een toegenomen biochemische vraag naar glycine in ratten, zoals gepostuleerd in patienten met episodische psychosen vergezeld gaand met meervoudige perceptuele stoornissen, psychotische reacties zou kunnen oproepen. De duur van de catalepsie werd gebruikt als een maatstaf voor psychotische reacties, en werd gemeten in porfyrische ratten na toediening van serine of glycine. Deze catalepsie werd waargenomen na zowel serine als glycine toediening in ratten die voorbehandeld waren met 2-allyl-2-isopropyl-acetamide (AIA), terwijl in ratten, voorbehandeld met lood + phenobarbital, dit fenomeen alleen gemeten kon worden na glycine toediening. Toediening van AIA aan ratten resulteerde in een sterk toegenomen excretie van porfobilinogeen (PBG) in de urine, terwijl in lood + phenobarbital voorbehandelde ratten een toename te zien gaven in excretie van delta-aminolevulinezuur (delta-ALA). De ratten voorbehandeld met lood + phenobarbital vertoonden 18 uur na deze injecties toegenomen serine-, en afgenomen glycine bloedspiegels. Er werden geen significante verschillen in bloedspiegels van deze aminozuren gevonden 24 uur na AIA toediening. In alle ratten, behalve diegene voorbehandeld met lood + phenobarbital, was de vorming van glycine verhoogd na serine toediening. Geconcludeerd werd dat het gevonden diermodel gebruikt kan worden om psychosen nader te bestuderen.

In Hoofdstuk III wordt de ontwikkeling beschreven van een snelle methode ter extractie vanuit humaan en rattebloedplaatjes en plaatjes arm plasma (PPP) en concentrering van tryptamine

(TA), 5-hydroxy-TA en 5-methoxy-TA. Hierbij werd gebruik gemaakt van reverse-phase C-18 kolommetjes voor monstervoorbereiding, in combinatie met een ionpaarvormend reagens. Met behulp van deze methode konden 1,2,3,4-tetrahydro-beta-carboline (THBC), 6-hydroxy-THBC en 6-methoxy-THBC, welke de respectievelijke reactieprodukten zijn na reactie van formaldehyde met de bovengenoemde biogene amines, eveneens worden geextraheerd, evenals beta-carboline (BC, norharman) en 1-methyl-beta-carboline (1-Me-BC, harman). Een HPLC methode, gecombineerd met fluorometrische detectie werd ontwikkeld voor de quantitatieve bepaling van deze stoffen in de orde van picomolen/ml bloed. De artefactuele vorming van beta-carbolines gedurende de extractie procedure was beneden de detectiegrens van de bepalingsmethode. 6-OH-THBC, 1-Me-BC en 5-HT bleken normale bestanddelen te zijn in menselijke bloedplaatjes, terwijl alleen 5-HT en 6-OH-THBC kon worden gedetecteerd in menselijk PPP. In bloedplaatjes en PPP van ratten kon alleen 5-HT worden gedetecteerd.

Porferie werd geinduceerd in volwassen mannelijke ratten, welke 24 uur hadden gevast, door subcutane injectie van 400 mg/kg allylisopropylacetamide (AIA), hetgeen is beschreven in Hoofdstuk IV. De porferie werd aangetoond door na AIA-injectie de 24-uurs urineexcretie te meten van delta-aminolevulinezuur (delta-ALA) en porfobilinogeen (PBG). De bloedspiegels van glycine, serine en een aantal andere aminozuren bleken verlaagd te zijn in porferische ratten, in vergelijking met controles. De intraperitoneale injectie van 2 mmol/kg serine, 24 uur na AIA toediening werd gebruikt als diermodel voor psychose, door 30 minuten na serine-injectie de duur van de catalepsie te meten, zoals is beschreven in Hoofdstuk II.De concentratie van 5 verschillende beta-carbolines in plaatjes rijk plasma (PRP) werd gemeten met behulp van bovengenoemde HPLC-fluorometrie-methode. Een toename in de norharman concentratie in PRP werd gemeten, varierend van 0.57 nanomol/l in controles, tot 1.88 nanomol/l in porferische ratten, aan welke laatste serine was toegediend. De duur van de catalepsie bleek exponentieel gecorreleerd met de norharman concentraties in PRP. Er werd geconcludeerd dat een toegenomen conversie van serine naar glycine verantwoordelijk zou kunnen zijn voor de toegenomen norharman biosynthese.

Zoals beschreven door Pepplinkhuizen (Proefschrift 1983), kon in een groep patienten, hersteld van episodisch psychotische aanvallen, konden deze symptomen binnen 2-4 uur weer worden opgewekt door orale toediening van serine. Deze waarneming ondersteunde de hypothese dat een toegenomen omzetting van serine in glycine resulteert in ophoping van $CH_2=FH_4$. Het is bekend dat $CH_2=FH_4$ niet-enzymatisch kan ontleden in tetrahydrofoliumzuur en

formaldehyde. Deze laatste stof kan reageren met indoleamines of catecholamines tot respectievelijk tetrahydro-beta-carbolines of tetrahydroisoquinolines. In dit hoofdstuk V wordt voor het eerst aangetoond dat de beta-carboline norharman aanwezig is in het bloedplasma van acuut psychotische patienten. Deze stof kon niet worden aangetoond in plasma van gezonde controles of van symptoomvrije patienten. De aanwezigheid van norharman in plasma van deze patienten zou kunnen betekenen dat deze stof een endogeen gevormde verbinding is met hallucinogene werking, verantwoordelijk voor een of meerdere symptomen van de desbetreffende psychose, o.a. angst.

In Hoofdstuk VI wordt geconcludeerd dat de aanwezigheid van norharman, welke verbinding op zichzelf farmacologisch buitengewoon actief is, een belangrijke vinding is op het gebied van de biologische psychiatrie. Het zou voor de eerste maal zijn dat een endogeen gevormde, mogelijk hallucinogene stof aangetoond is in episodisch acuut psychotische patienten, en een mogelijk oorzakelijk verband vertoont met een of meerdere symptomen. Deze resultaten werden ondersteund door het feit dat dezelfde verbinding werd gevonden in ratten tijdens gedragsstoornissen na op chemische wijze de omzetting van serine naar glycine te verhogen, welk laatste verschijnsel waarschijnlijk ook plaatsvindt in voornoemde patienten.

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CURRICULUM VITAE

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In 1970 behaalde ik mijn 5-jarig HBS-B diploma aan het Keizer Karel College te Amstelveen, waarna de studie scheikunde met hoofdvak Biochemie, gevolgd op het instituuut van Prof. Planta, in dat jaar een een aanvang nam. Tijdens deze studie aan de Vrije Universiteit te Amsterdam ben ik gedurende drie jaar werkzaam geweest als student-assistent ten behoeve van studenten-practica. In 1979 behaalde ik mijn doctoraal examen biochemie. Vanaf mei 1980 tot mei 1984 heb ik het grootste deel van dit in dit proefschrift beschreven promotieonderzoek verricht onder leiding van Prof. Dr. J. Bruinvels, op de afdeling Farmacologie aan de Erasmus Universiteit te Rotterdam. Vanaf mei 1984 tot en met oktober 1984 heb ik additioneel onderzoek verricht op de afdeling Analytische Chemie aan de Vrije Universiteit te Amsterdam, onder leiding van Prof. Dr. U.A.Th. Brinkman. Sedert half augustus 1985 ben ik werkzaam als programmeur bij het software-huis CPP Nederland BV te Weesp.