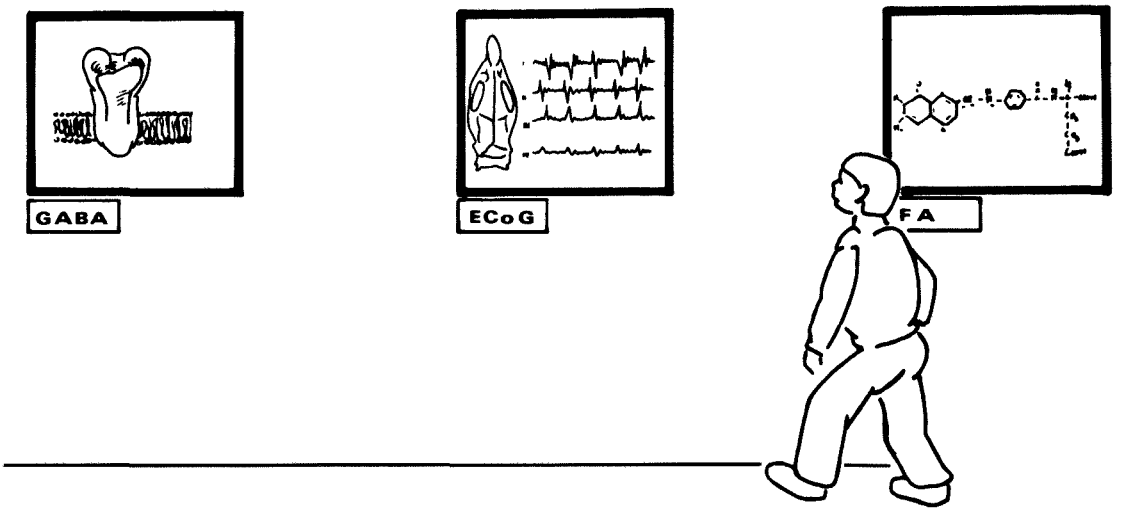
FOLIC ACID, EPILEPSY and the $GABA_A$ RECEPTOR COMPLEX

Complementary in vivo and in vitro studies concerning their interrelationships



Clementina Maria van Rijn

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$GABA_A$ RECEPTOR COMPLEX

Complementary in vivo and in vitro studies
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Een wetenschappelijke proeve op het gebied van de
Geneeskunde en de Tandheelkunde.

PROEFSCHRIFT

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Met Jan Pieter samen.

Voor mijn moeder.

In de nagedachtenis aan mijn vader.

SUMMARY

In the **INTRODUCTION** (ch. 1) the three elements which make up the title of this thesis will be treated briefly. In doing so the central problem will be presented, namely, "what biochemical mechanism is responsible for the epileptogenic actions of folic acid?"

*In the **PROMENADES** we explain the approach to the central problem of the thesis and the connections between the various chapters. Please refer to these promenades for a summary of the various chapters (in english or in dutch)*

In the **CONCLUSION** (ch. 9) new questions arising from the results of the experiments will be discussed. These questions do not only concern the subject of this thesis, but deal with both the theory of receptor-binding-studies in general and with the $GABA_A$ complex in particular as well.

SAMENVATTING

In de **INLEIDING** (hfdst. 1) wordt op de drie begrippen die samen de titel van dit proefschrift vormen ingegaan. Hierbij wordt ook de centrale vraagstelling die aan dit werk ten grondslag ligt gepresenteerd, namelijk "door welk biochemisch mechanisme is foliumzuur in staat om epileptogene verschijnselen te veroorzaken?".

*In de **PROMENADES** wordt de aanpak van de vraagstelling en de samenhang van de verschillende hoofdstukken toegelicht (in het engels en in het nederlands). Voor een samenvatting van de afzonderlijke hoofdstukken verwijs ik u graag naar de promenades*

In de **CONCLUSIE** (hfdst. 9) worden nieuwe vragen die opgeroepen worden door de resultaten van de uitgevoerde experimenten aangestipt. Deze vragen hangen niet alleen samen met de vraagstelling in dit proefschrift, maar hebben ook betrekking op zowel de theorie van receptor-binding-studies in het algemeen als op het $GABA_A$ complex in het bijzonder.

CONTENTS PAGE

THE BLIND MEN AND THE ELEPHANT

It was six men of Indostan
To learning much inclined
Who went to see the Elephant
(Though all of them were blind),
That each by observation
Might satisfy his mind

The First approached the Elephant
And happening to fall
Against his broad and sturdy side
At once began to bawl
"God bless me! but the Elephant
Is very like a wall!"

The Second feeling of the tusk
Cried "Ho! what have we here
So very round and smooth and sharp?
To me 'tis mighty clear
This wonder of an Elephant
Is very like a spear!"

The Third approached the animal,
And happening to take
The squirming trunk within his hands
Thus boldly up and spake
"I see" quoth he "the Elephant
Is very like a snake!"

The Fourth reached out an eager hand
And felt about the knee
"What most this wondrous beast is like
Is mighty plain" quoth he
"'Tis clear enough the Elephant
Is very like a tree!"

The Fifth who chanced to touch the ear
Said 'E'en the blindest man
Can tell what this resembles most,
Deny the fact who can
This marvel of an Elephant
Is very like a fan!"

The Sixth no sooner had begun
About the beast to grope,
Than seizing on the swinging tail
That fell within his scope,
"I see" quoth he "the Elephant
Is very like a rope!"

And so these men of Indostan
Disputed loud and long
Each in his own opinion
Exceeding stiff and strong
Though each was partly in the right
And all were in the wrong!

John Godfrey Saxe,
American Poet 1816-1887

1.1 EPILEPSY.

Epilepsy in human is a common disorder affecting 6.25/1000 people [6] Epilepsy is not a clearly defined disorder The term is considered to describe chronic brain syndromes of various etiology characterized by recurrent convulsive and non-convulsive seizures due to excessive discharges of cerebral neurons attended with a variety of clinical manifestations [5, 32]

The epilepsies may be divided into two main groups the generalized epilepsies and the partial or focal epilepsies

- The generalized epilepsies are characterized by an initial disturbance of consciousness accompanied with other symptoms such as convulsions or absences The EEG exhibits synchronous discharges from both hemispheres
  Partial epilepsies may exhibit localized symptoms of motoric sensoric autonomic or mental character attended with abnormalities on the EEG originating from a circumscribed part of the brain The partial seizures may develop into generalized seizures

In addition to the division described above, epilepsy may be described as idiopathic or as symptomatic

- Idiopathic epilepsy implies that the cause of the disorder is unknown
- Symptomatic epilepsy is due to some demonstrable brain disease (e g congenital cerebral defects, intracranial or general infections, intoxications, cerebral tumors vascular disorders or cerebral degeneration) [12]

Whether the epilepsies are due to a single common etiological cause is unknown [3, 6, 7] Aspects of possible etiological factors have been reviewed recently [3 7] Only a start has been made to unravel the cellular and molecular mechanisms of the epilepsies Two systems are thought to be of particular importance in the epileptogenesis the inhibitory GABA system and the excitatory glutamate system [1, 8, 16, 17, 18, 23 29, 33, 34, 35]

Animal models of epilepsy are of great importance to the search for the basic neuronal disfunction underlying the disease as well as to the search for new

effective antiepileptic drugs [9 14, 19, 20 26, 27 30] The animals may be affected spontaneously or the seizures may be invoked by sensoric stimulation [2, 4, 15, 22, 24, 25, 31] Moreover, epileptic phenomena can be induced in animals, e g by convulsive drugs, by electrical stimulation (by electroconvulsive shocks and by 'kindling') [11] Chemically, seizures are often induced by compounds alien to the body such as pentylenetetrazol (Cardiazole), penicillin and kainic acid [10, 13, 21] Some endogenous substances can induce seizures as well e g glutamate, aspartate, and folic acid, all in high concentrations [3, 28] Glutamate and aspartate are known excitatory neurotransmitters In contrast, the mechanism underlying the epileptogenic effects of folic acid is not known

In this thesis we describe our investigations on the mechanism of the epileptogenic action of folic acid In the studies described, an animal model of chemically induced, partial epilepsy with elementary motor symptoms is used

1.2 FOLATES.

Folates in general.

Folic acid owes its name to its abundant presence in green leafy vegetables, especially spinach (folio means leave), but it is present in nearly all food substances [5] Folic acid is reduced in the body into a series of derivatives [6] Folic acid and its derivatives are collectively called the folates Folates are needed in biological syntheses they are involved as coenzymes in nearly all those metabolic functions in which there is a transfer of one-carbon units

Folates are commonly known because of

- their role in megaloblastic anaemia due to their involvement in e g DNA synthesis [44] Adequate folate availability is a precondition for cell proliferation [6]
- the role of folate antimetabolites in cancer therapy [4] Methotrexate blocks dihydrofolate reductase, resulting in a depletion of reduced i e metabolically active folates
- the bacteriostatic properties of sulfonamides [54] Mammals do not synthesize folic acid it is a vitamin Bacteria must synthesize folic acid them-

selves. This synthesis can be blocked by sulfonamides, which are antimetabolites of paraaminobenzoic acid (PABA), which is one of the components of folic acid.

In addition folates are able to induce epileptic phenomena when they have penetrated into the brain [26]. It is not likely that a mechanism associated with the one-carbon transfer properties of the folates can account for the epileptogenic effects, as the antimetabolite Methotrexate is able to induce convulsions as well [38, 39]. Methotrexate has no one-carbon transfer properties.

Chemical structure of the folates.

Folic acid (Pteroylglutamic acid, Vitamin Bc, $C_{19}H_{19}N_7O_6$, Mol. wt 441), is composed of 2-amino-4-hydroxy-pteridine, paraaminobenzoic acid and glutamic acid [7]. (Structural formula see folder at the end of the thesis.) Folates are present in various metabolically active reduced forms, such as 5-formyl-tetrahydrofolate (5-HCO-H_4folate), dihydrofolate (H_2folate) and 5-methyl-tetrahydrofolate (5-CH_3-H_4folate).

The most abundant form of folates in the body is that in which more than one glutamate unit is present in the molecule. The glutamate units are linked chainlike by amide bonds to the γ-carboxyl group of the preceding glutamate [35].

Folates in the central nervous system.

In rats and in humans the concentration of folates in the CSF (cerebro-spinal fluid) is substantially higher than the concentration in plasma [8, 50, 59]. Reduced folates are transported from the blood into the CSF and oxidized folates are transported out of the CSF back into the blood by a carrier-mediated transport system located in the plexus chorioideus [21, 49, 55]. These transport processes at the blood-brain barrier help to maintain a reduced folate homeostasis in the brain. No oxidized folate (i.e. folic acid itself) was detected in brain tissue. 5-Methyl-tetrahydrofolate and tetrahydrofolate are the predominantly occurring monoglutamate-folates in the (rat)brain. The monoglutamate-folates do not account for more than 10 % of the total folate pool in the brain. About 70 % of the endogenous folates in the brain of the rat are pentaglutamates or folates with greater chain length [10]. The available data of total folate concentrations should be interpreted with this in mind.

Folate concentrations

Total folate	Human	serum	5 - 12 ng/ml	[30]
		CSF	14 - 31 ng/ml	[8]
		cortex grey	+ 400 ng/g ww	[61]
		cortex white	± 200 ng/g ww	[61]
		liver	3000 ng/g ww	[22]
Total folate	Rat	serum	53 - 190 ng/ml	[6]
		brain	360 630 ng/g ww	[10]
		liver	12000 ng/g ww	[20]
5-CH_3-H_4-folate	Rat	cortex	± 44 ng/g ww	[30]

Folates and epilepsy.

Interest in the convulsive action of folates was first raised by the observation of lowered folate serum levels in patients receiving anticonvulsant medication [3 8 17 43, 44] It was hypothesized that the anticonvulsant action of the medication might result from a folate-lowering effect, and thus that folate derivatives might have convulsive properties [11, 12, 37] In humans the proposed convulsive effect of folates, still the subject of many studies is neither proven nor dismissed [8 32, 43, 45 60] In animals it has been shown that folate derivatives do indeed have convulsive activity [1 25 27, 28, 37, 51, 53] The biochemical mechanism of this activity is unknown

With this thesis we hope to contribute to the elucidation of this mechanism

Synopsis of previous biochemical studies.

A number of mechanisms have so far been proposed to underlie the convulsive action of the folates

- A direct action of folates, probably on the GABA receptor was proposed in the early seventies [16, 24 51] It was not until 1985 that some electrophysiological indications for this GABA-folate interaction hypothesis were published [42]
- In the mean time folates have been reported to inhibit the high affinity uptake of glutamate [46], and to inhibit the uptake of a variety of neurotransmitters, GABA included, as well [9]

- Folates are able to inhibit the enzyme GAD (glutamate decarboxylase) [58] but the rank order of potency does not correlate with the epileptogenic actions (M G P Feenstra, personal communication)
- In 1980 it was reported that 5-methyl-tetrahydrofolate is a potent displacer of the glutaminergic compound ^{3}H-kainic acid from its specific receptor sites [48] It was proposed that the folate derivative might be the endogenous ligand of the kainate receptor This would suggest that folates in the brain may function as excitatory neurotransmitters [23, 29, 52] This finding however was not confirmed in other receptor binding studies [19], nor in electrophysiological studies [2, 18 19, 31, 36], nor in neurotoxicity studies [15 33 34, 40, 41, 47 56 57]

As noted above a direct action of folates on the GABA receptor was suggested in 1973 [16] Collingridge indicated that folates increased the probability of neuronal discharge [14] Clifford suggested that the mechanism of action of folates was more likely to be the result of disinhibition than of direct excitation [13] Otis, finally, showed that the application of folic acid to neurons results in a reduction of the GABA-mediated inhibitory postsynaptic potentials, and in a reduction of the response to iontophoretically applied GABA [42] These results suggest that folic acid exerts its action by a disinhibitory mechanism, i e by antagonizing the postsynaptic action of GABA

1 3 GABA RECEPTORS.

Receptors in general

Receptors may be defined as proteins to which a compound may bind reversibly, in such a way as to induce a conformational change in the protein which ultimately leads to a physiological response in the system Response inducing substances are called agonists Drugs that are able to bind to the receptor without inducing a response are called antagonists

Neurotransmitter receptors are located in the plasma membrane of neurons They can be divided into two classes [36]

Class 1 The ligand-gated receptor ion channels These receptors induce a fast conductance change They do not need a second messenger system for this effect

Class 2 Receptors coupled to a second messenger system and/or a G protein

A number of neurotransmitter substances have been identified in the mammalian central nervous system Among these GABA is one of the most abundant [30] Anatomical studies suggest that GABA is predominantly located in small interneurons scattered throughout the central nervous system [26] Agents capable of potentiating GABAergic transmission may be expected to have a variety of biological effects anticonvulsant, antidepressant, anxiolytic, hypnotic and analgetic effects have been reported [5, 7, 35] However apart from differences in anatomical location, this variety of effects of GABAergic drugs may be due to neurochemical differences one may think e g of differences in sensitivity of the GABA metabolizing enzymes Another possibility is that all GABA receptors may not be pharmacologically and functionally equivalent there may be distinct subgroups of GABA receptors which may be selectively manipulated At least two such subgroups are defined, and it is not likely that each group consists of a homogenous population [1, 2, 5, 13]

- The $GABA_A$ receptors are linked to chloride channels such that receptor activation by GABA leads in general to an inward movement of Cl^--ions, resulting in a hyperpolarization (i e inhibition) of the postsynaptic cell The $GABA_A$ receptors belong to the class 1 receptors Bicuculline antagonizes this action of GABA, whereas muscimol mimics it Baclofen has no effect on these $GABA_A$ receptors
- The $GABA_B$ receptors activation of these receptors by GABA is thought to lead to a reduction of evoked excitatory neurotransmitter release resulting in a decreased excitation of the postsynaptic cell [5] $GABA_B$ receptors belong to the class 2 receptors as they modulate adenylatecyclase activity via an interaction with a GTP binding protein [38] These receptors are activated by (-)-Baclofen and GABA Bicuculline does not bind to the $GABA_B$ receptors

The receptor binding studies described in this thesis are concerned exclusively with the $GABA_A$ receptor complex of the rat brain

The $GABA_A$-receptor-complex-Cl^-channel.

See figure 1 (next page) and the folder at the end of the thesis

Pharmacological and ligand binding studies have identified a number of binding sites on the $GABA_A$ receptor complex

- The GABA agonist/antagonist site [5, 30, 31 36 55] This site is likely to be heterogeneous [3, 11, 21, 42]
- The benzodiazepine site, which may be heterogeneous too [25 36, 42, 47]
- The convulsant or channel gating site where agents like picrotoxin and TBOB (t-butylbicycloorthobenzoate) will bind [15, 34, 43, 52] Probably this site is heterogenous as well [32, 49]
- The depressant site, recognizing barbiturates [53]
- Sites binding the channel-permeating anions [10, 36]

Each of these types of ligand sites can interact allosterically with one or more of the other sites, resulting in a network of interactions [3, 4, 9, 17 19 20, 22, 23, 29, 40, 50, 51 54]

Recently, the protein structure of the complex has been determined [1, 14, 41] The complex consists of two subunits α and β, with a stoichiometry of $\alpha_2\beta_2$ The α units carry the benzodiazepine recognition sites, whereas the GABA recognition sites are located on the β units The α subunit alone exists in at least three different varieties [18] This finding confirms the heterogeneous nature of the receptor/Cl^- channel complex Both binding sites for GABA must be occupied with agonists in order to induce channel opening [47, 48] Binding of ligands to the benzodiazepine site influences the frequency of channel opening induced by GABA Benzodiazepine agonists increase the action of GABA, inverse agonists decrease the influence of GABA [16, 27, 28] Binding of agents to the convulsant site blocks the GABA-activated channel, whereas binding of agonistic ligands to the depressant site prolongs the duration of aperture-opening of the GABA-activated channel [5, 33]

In addition to the ligand-receptor interactions named so far, some other effects on the complex have been described

- A number of compounds with diverse chemical structures have been shown to modulate the GABA receptor function [8, 12, 44, 45, 46] Alcohols, a number of anaesthetics (e g etomidate) and steroids (e g progesterone) all affect the $GABA_A$ receptor function [6, 24, 33] Several compounds which are known

noncompetitive blockers of other class 1 receptors (the nicotinic acetylcholine receptor and the N methyl-d-aspartate subtype of the glutamate receptor) also inhibit the GABA gated Cl^- channel (e g phencyclidine D-tubocurarine) [37]

- The presence of membrane phospholipids and the nature of the receptor-membrane interactions are essential to the integrity of the convulsant site [36, 39]

In summary the $GABA_A$-receptor-complex-Cl^- channel is an oligomeric membrane-bound protein complex with allosteric and modulatory sites

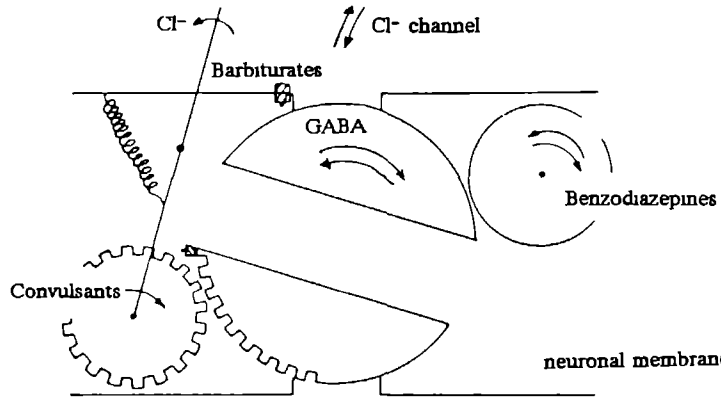

Fig1 ***The $GABA_A$ receptor complex:***
The control of the Cl^- channels has been represented by a revolving tap mechanism Binding sites
GABA and GABA agonists rotate the 'tap' into the open position, whereas GABA antagonists oppose this rotation The effects of drugs which affect the channel mechanism but which do not act at the GABA recognition site are represented by adjacent wheels These wheels may be considered as allosteric sites

- *In the model, the influence of the benzodiazepine agonists, i e an increase in the frequency with which the GABA-operated Cl^- channels open, is represented by an increase in the rate at which the channel wheel rotates caused by an adjacent wheel*
- *The cogwheel represents the convulsant binding site When convulsants bind to this site the rotation produced prevents the GABA induced increase in Cl^- conductance*
  The influence of the convulsants may depend on the external Cl^- concentration and this action is represented by the pivot-lever
- *The influence of barbiturates has been represented by a 'latch' mechanism which on activation keeps the channel in open position*

(Reproduced (modified) from N G Bowery, et al, Neuropharmacology, 23 (1984) 219-231, with permission)

The research described in this thesis deals with the biochemical mechanism of the epileptogenic action of folic acid and its derivatives Two different experimental approaches were used

- *in vivo intracerebral application of convulsants in the rat*
- *in vitro receptor binding assays on rat brain membranes*

The in vivo studies are presented in Part 1 of this thesis (chapters 2, 3, and 4) whereas Part 2 contains the in vitro investigations (chapters 5 6, 7 and 8) Conclusions on the possible relationship between the in vivo and in vitro effects of the folates are discussed in chapter 9

In the following paragraphs of this PROMENADE the various chapters are briefly highlighted to show the interrelationship between the subjects and the development of our investigations

Part 1: In vivo: Chapters 2,3,4.

The aim of the investigations presented in this part was to answer the following questions

1) ***Does folic acid, when injected into the neocortex, induce the same clinical course and electrophysiological semiology as (classes of) convulsants with better known mechanisms of action?***

Phenomena caused by folic acid injection are compared to the effects of kainic acid, carbachol, neostigmine bicuculline penicillin and strychnine (chapter 2) In chapter 3 seizures induced by baclofen are reported It turned out that in the neocortex folic acid epilepsy is comparable to that induced by GABAergic inhibitory compounds, especially picrotoxin and bicuculline On the basis of this result we developed the hypothesis that folic acid might have a GABAergic inhibitory action As it is known that the part of the prepiriform cortex known as "area tempestas" (region of storms) is very sensitive to bicuculline, we considered the comparison of folic acid to bicuculline in this area to be a good test for the resemblance of the two drugs The next question was therefore

2) Will the pattern of epileptogenicity as observed in the neocortex be found in the "area tempestas" as well?

The convulsive effects of folic acid, bicuculline and kainic acid injected into the prepiriform cortex are described and compared (chapter 4)

Part 2 In vitro: Chapters 5,6,7,8.

Starting point for the in vitro studies described in part 2 of this thesis was the GABAergic inhibiting mechanism of the substances mimicking folic acid in the neocortex. Our aim therefore was to answer the following questions

3) Do folates affect the $GABA_A$ complex in such a way as to account for the epileptogenic phenomena?

The effects of four folates on three different binding sites on the $GABA_A$ receptor complex were investigated (viz the high affinity GABA binding site, the benzodiazepine binding site and the convulsant site) and compared to the ability of the folates to induce epileptic phenomena in vivo (chapter 6). An enhancement of the binding of ^3H-TBOB to the convulsant site was found.

As the radioligand ^3H-TBOB was only recently introduced, we have included in the preceding chapter 5 our determination of the binding characteristics of this radioligand.

The enhancement of ^3H-TBOB binding by the folates led us to try to answer the following questions

4) What is the mechanism of the enhancement of the binding of ^3H-TBOB by folates?

The influence of folic acid on the binding of the convulsant ^3H-TBOB is the subject of chapter 7.

5) What is the site of interaction of the folates with the $GABA_A$ complex?

In chapter 8 the in vitro effects of the folates, bicuculline and βCCE are compared.

Het onderzoek dat in dit proefschrift beschreven wordt betreft het biochemisch mechanisme dat ten grondslag ligt aan de epileptogene werking van foliumzuur en zijn derivaten. Er werden twee experimentele opzetten gebruikt

- *in vivo intracerebrale toediening van convulsieve stoffen in de rat*
- *in vitro receptor-binding-studies aan membranen van rattehersenen*

De in vivo studies worden beschreven in deel 1 van dit proefschrift (hoofdstukken 2, 3 en 4). Deel twee bevat de in vitro studies (hoofdstukken 5, 6, 7 en 8). Een mogelijke relatie tussen de in vivo en in vitro effecten wordt o.a. besproken in hoofdstuk 9.

In de volgende paragrafen van deze promenade worden de hoofdstukken van dit proefschrift kort toegelicht. Dit om de samenhang tussen de onderdelen te tonen en de ontwikkeling van ons onderzoek te schetsen.

Deel 1: In vivo: Hoofdstukken 2,3,4.

Het doel van de hier beschreven proeven was een antwoord te vinden op de volgende vragen:

1) ***Veroorzaakt foliumzuur, wanneer dat geïnjecteerd wordt in de neocortex, dezelfde clinische en electrofysiologische verschijnselen als (klassen van) convulsiva waarvan het werkingsmechanisme beter bekend is?***

De verschijnselen die veroorzaakt worden door foliumzuur werden vergeleken met de effecten veroorzaakt door kainezuur, carbachol, neostigmine, bicuculline, penicilline, strychnine (hfdst. 2) en baclofen (hfdst. 3). In de neocortex bleek de door foliumzuur geïnduceerde epilepsie vergelijkbaar te zijn met de epilepsie veroorzaakt door GABAerge inhibitore stoffen, met name picrotoxine en bicuculline. Op basis van dit resultaat stelden we de hypothese op dat foliumzuur een GABA inhibitore werking zou kunnen hebben.

Het is bekend dat het deel van de prepiriforme cortex dat "area tempestas" wordt genoemd ("het gebied van de stormen") zeer gevoelig is voor bicuculline. Om onze hypothese te testen in vivo hebben wij daarom ook in dit gebied het effect van de stoffen vergeleken. Onze vraag was:

2) **Wordt het patroon van epileptogeniciteit zoals we dat zien in de neocortex ook waargenomen in de "area tempestas"?**

De verschijnselen veroorzaakt door het injecteren van foliumzuur bicuculline en kainezuur in de prepiriforme cortex worden beschreven en vergeleken in hfdst 4

Deel 2: In vitro: Hoofdstukken 5,6,7,8.

Het uitgangspunt voor de in vitro studies, beschreven in deel twee, was dat die stoffen, die geinjecteerd in de neocortex de effecten van foliumzuur imiteren, een $GABA_A$ inhiberend werkingsmechanisme hebben Ons doel was daarom de volgende vragen te beantwoorden

3) **Hebben folaten een zodanig effect op het $GABA_A$ complex dat dit de epileptogene verschijnselen kan verklaren?**

Onderzocht werden de effecten van vier folaten op drie verschillende bindingsplaatsen van het complex (namelijk de hoge affiniteit GABA plaats, de benzodiazepine plaats en de convulsieve plaats) De gevonden in vitro folaat effecten werden vergeleken met de epileptogeniciteit van de vier folaten in vivo (hfdst 6) Het bleek dat folaten de binding van het radioligand ^{3}H-TBOB aan de convulsieve plaats op het complex verhogen

Omdat het radioligand ^{3}H-TBOB pas kort geleden geintroduceerd is hebben wij in het voorafgaande hoofdstuk 5 de bindingskarakteristieken beschreven zoals wij die hebben gemeten

De gevonden verhoging van de ^{3}H-TBOB binding o i v de folaten leidde tot de volgende vragen

4) **Door welk mechanisme verhogen de folaten de ^{3}H-TBOB binding?**

De invloed van foliumzuur op de bindingskarakteristieken van het convulsivum ^{3}H-TBOB wordt beschreven in hoofdstuk 7

5) **Met welke plaats op het $GABA_A$ complex heeft foliumzuur een interactie?**

In hoofdstuk 8 wordt het in vitro effect van foliumzuur vergeleken met dat van bicuculline en dat van βCCE

PARTIAL MOTOR EPILEPSY INDUCED BY INTRA-NEOCORTICAL ADMINISTRATION OF FOLIC ACID IN FREELY MOVING RATS COMPARISON WITH GABA-ERGIC INHIBITORY COMPOUNDS AND DIRECT EXCITATORY COMPOUNDS

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T J A M van der Velden, A J M M Beekman and O R Hommes

SUMMARY.
Folic acid can evoke epileptic phenomena when it penetrates into the brain The biochemical background of this action is unknown A direct action of folic acid on synaptic receptors, specific the inhibitory receptors, has been proposed earlier Following this suggestion the epileptic phenomena caused by folic acid are compared to those of disinhibitory drugs (i e bicuculline, strychnine, penicillin and picrotoxin) and to those of excitatory substances (i e kainic acid, carbachol and neostigmine) The epileptic phenomena induced by folic acid resemble closely those induced by the disinhibitory compounds, but differ in many respects from those induced by the direct excitatory drugs These findings support the suggestion that folic acid might block the inhibitory system

INTRODUCTION.

Folic acid and several of its reduced derivatives have been shown to have an epileptogenic action on the mammalian brain [24, 34, 43] High doses of these compounds are needed to produce epileptic effects after peripheral administration (225mg - 625mg Na-folate/kg bodyweight) near the LD_{50} (450 mg/kg), but these doses can be considerably reduced when there is direct access to brain tissue (75 mg/kg)[25 33] The epileptogenic potential of folic acid has been studied in our department and used to develop a test model for anticonvulsant drug action on partial motor epilepsy, comparable to the penicillin model [1] In this thesis we used a modification of the model to investigate the mechanism of folic acid induced epilepsy

A major impetus for recent studies on the mechanism of the folic acid actions was the suggestion that folic acid might have a similar mechanism as kainic acid a glutamate analogue with excitatory and neurotoxic properties [2 11 27 30 35, 40 41, 44] However, the neurotoxic properties of folic acid seem to be different from those of kainic acid folic acid appeared to reproduce the distant but not the local neurotoxic effects of kainic acid [6, 16, 20, 26, 31, 36, 47, 48] More over, direct excitatory effects of the folates are weak [2, 14, 15, 17, 19] In addition, it has been suggested that a disinhibitory action might be the basis of the observed epileptic manifestations of folic acid [15, 17, 22, 38]

The endogenous presence of reduced folates in the brain, blood and peripheral organs, and the presence of all kinds of folates in food [7] is a highly interesting fact in view of the epileptogenic action In addition to its presence in food, high amounts of folic acid are present in vitamin preparations [13] and folates have been proposed in the prevention of neural tube defects [42]

The observation that folic acid showed a potentiation of epileptogenic kindling [32] suggests that folic acid, when repeatedly ingested or endogenously liberated, might exert excitatory effects

In this study we describe behavioral and electrographical effects of intracortical injections of low doses of folic acid and defined qualitative and quantitative measurements of folic acid effects and compared the action of folic acid to that of a number of other epileptogenic substances with better known mechanisms of action

MATERIALS AND METHODS.

Subjects.

Male Wistar albino rats (CPB/TNO, Zeist, The Netherlands) were used, with a weight of 200 ± 10 g at time of surgery for those for observational experiments, and 260 ± 10 g for those for electrocorticographical experiments The animals were individually housed and allowed access to food and water ad libitum A 12 h light, 12 h dark cycle was maintained, light on at 7 a m The experiments took place in the light phase

Surgery

The animals were anaesthetized by pentobarbital (45 mg/kg ip) and atropine (1mg/kg sc) A polyethylene cannula (outer diameter 0 8 mm, inner diameter 0 4 mm) [9] was implanted through a drill hole in the skull 1 4 mm to the right of bregma, where the sensorimotor cortex of the left hindleg is situated The cannula was fixed by acrylic cement The tip of the cannula, cut to an edge of 45° to facilitate the penetration of the dural membranes was 2 mm beneath the upper surface of the skull histological examination revealed that the tip was in lamina IV or V of the cortex The cannula could be connected to a flexible injection system This permitted free movement during administration of the drugs

For electrocorticographical recordings the animals received 4 epidural (and 2 nasal reference) electrodes on the skull as well positions related to the bregma anterior 0 0 mm, lateral 3 6 mm, posterior 6 0 mm, lateral 4 0 mm (references anterior 6 0 mm, lateral 1 5 mm) The electrodes, stainless steel screws 1 mm x 2 mm, were connected to a minisocket (MTA, Cannon ITT) and embedded in acrylic cement Free movement remained possible during ECoG registration The animals were left to recover from surgery for 5 7 days

Clinical observations.

The drugs, dissolved in distilled water, were injected through the cannula in a volume of 0 5 - 2 0 µl, with a rate of 0 5 µl/min One test per day was conducted

The animals were observed for 1 5 h following injection of the drug For each 5 min period the maximum values of the intensity of the single myoclonic jerks and the spread of the jerks over the body (extension),(table 1, when doubt, half points could be adjudged), the number of the seizures and the duration of the phenomena were noted In addition, the total number of the jerks was counted in four periods of 5 minutes, with intervals of 10 minutes (10-15, 25-30, 40-45 and 55-60 minutes) All registrations were done on animals that were used for the first time after surgery

Electrocorticographical registrations.

The ECoGs were recorded on a Siemens Elema 8-channel mingograph The amplification filter had an upper limit of 15 Hz and a time constant of 1 2 sec Each ECoG registration was started at least 0 5 h in advance of injection of the drug

in order to have a sufficient duration of baseline registration A marker was connected to one of the channels to allow registration of the jerks by an observator who could not see the recordings on the paper

Folic acid concentration determinations.

Brain folic acid concentrations after intracortical injections were determined with HPLC using a modification of the method of Lankema et al [29] At selected times after injection, a column of cortical tissue around the cannula tract was excised and divided into three parts upper (containing cortex laver I II) middle (laver III-V) and lower (laver VI and part of the corpus callosum) The pieces of tissue were frozen on dry ice and stored at - 80 °C The tissue was homogenized in 1 ml distilled water and 0 1 ml 0 1 % ascorbic acid solution After 3 min 1 ml of a 10 % solution of trichloracetic acid in 0 1 M HCl was added After centrifugation for 5 min at 2000 g, 1 ml of the supernatant was injected on a 15 cm Nucleosil 5C18 column (Chrompack, Middelburg, The Netherlands) A Waters M 45 solvent delivery system was used at a flow rate of 0 7 ml/min The analytical column was protected by a pellicular reversed phase precolumn (Chrompack) The eluent was 0 015 M citrate/phosphate buffer (pH 4 95 by addition of HCl) with 1 mg/l sodium azide and 13 % methanol A Schoeffel 770 spectrophotometric detector was operated at 280 nm The retention time of folic acid was about 9 min Blank samples showed no peak at this retention time Recoveries of folic acid were 85-95 % The detection limit was about 0 1 µg/g (0 2 nmol/g) in brain tissue

Chemicals.

All chemicals were obtained from Sigma

RESULTS 1 : Clinical effects of the drugs.

Folic acid.

Folic acid produced partial motor epileptic phenomena when injected into the right sensorimotor cortex at a dose of 2 nmol or more (fig 1,2) The first visible signs were jerks of the left hindlimb At the 5 nmol dose, epileptic phenomena were observed at 7 out of 8 rats (fig 1) or at 7 out of 7 rats (fig 3) The signs

began 5 min after the start of the injection (median value, range 3-8 min n=7) The intensity of the jerks increased with time, from a barely visible muscular jerk to a full contraction of the limb and a complete lift of the foot (fig 1) During this 'developmental' phase the frequency of the visible jerks increased as well and the jerks spread from the hindlimb only (extension class 1) to the left forelimb (class 2) and sometimes to the left vibrissae and ear (class 3)

Apart from the myoclonic jerks some focal and generalized seizures were seen, lasting approximately 10 seconds The focal seizures were characterized by a rhythmic succession of jerks, with a frequency of 2-3 Hz and with a larger extension and greater intensity than during the interictal periods At the generalized seizures a clonus of the whole body was observed No other behavioral abnormalities were observed consciousness was unimpaired and grooming behavior was normal (the generalized seizures excepted)

The duration of the motor symptoms after this dose of 5 nmol was 35 - 40 min The end phase generally set in with a rapid, sometimes abrupt, decline in intensity and frequency (fig 2) and a prolonged fading out with irregular jerks

Higher doses (10 -20 nmol) produced an increase in all measured parameters Thus, the intensity, extension and duration increased and seizures were more common (fig 1-3) However the delay of 5 min after the injection did not change Animals injected with 20 or 30 nmol sometimes had an increased susceptibility to seizures Than, seizures occurred in a rapid succession and could easily be induced by a sound or by touching the animal, stimuli that otherwise had no effect on the course of the events

Bicuculline.

Bicuculline-methylchloride produced partial motor epilepsy which was in many respects similar to the syndrome induced by folic acid (fig 3) However, it was more potent in that 10 times lower doses were active At the lowest dose tested, 0 1 nmol, in some animals visible jerks with irregular frequency, low intensity and of a short duration were observed This contrasts with the 10 times higher dose of folic acid (1 nmole) which did not produce any visible jerks in the 4 tested animals Like folic acid, bicuculline showed a dose dependent increase in all measured parameters (fig 3) The dose response curve was however less steep and the duration did not increase above a value of approximately 35 min

Kainic acid.

Kainic acid was injected in doses varying from 0.02 to 20 nmol. Although limb jerks were occasionally observed, reproducible motor signs as shown by folic acid and by bicuculline were never seen. The same animals tested later did show the familiar responses to folic acid though. Kainic acid induced variable signs reflecting the epilepsy which this compound elicits in other (limbic) brain regions such as the hippocampus and the amygdala: sniffing, head jerking, jaw movements, freezing and clonic seizures of both forelimbs were observed [47].

The phenomena following penicillin, picrotoxin, strychnine, carbachol and neostigmine were observed in combination with ECoG recordings only and will be described there.

Table 1. Classification of the myoclonic contractions.

Extension (= spread) Class/visible motor effect in:	Intensity Class/motor effect resulting in:
1 only left hindlimb	1 jerking without lifting the limb
2 left hindlimb and forelimb	2 jerking with lifting the limb
3 both left limbs + face	3 associated axial turning
4 contralateral	4 rolling on the back

Fig. 1 see next page

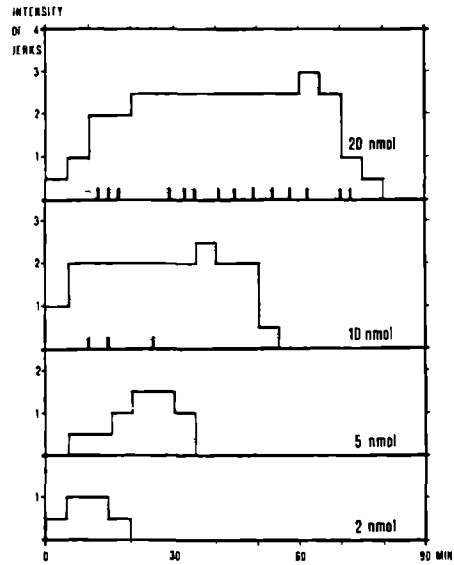


Fig. 2. Representative time/intensity diagram for the 4 tested doses of intracortical injected folic acid. The maximum intensity of the jerks (see Materials and Methods) was determined in 5 min periods. Short vertical bars indicate seizures.

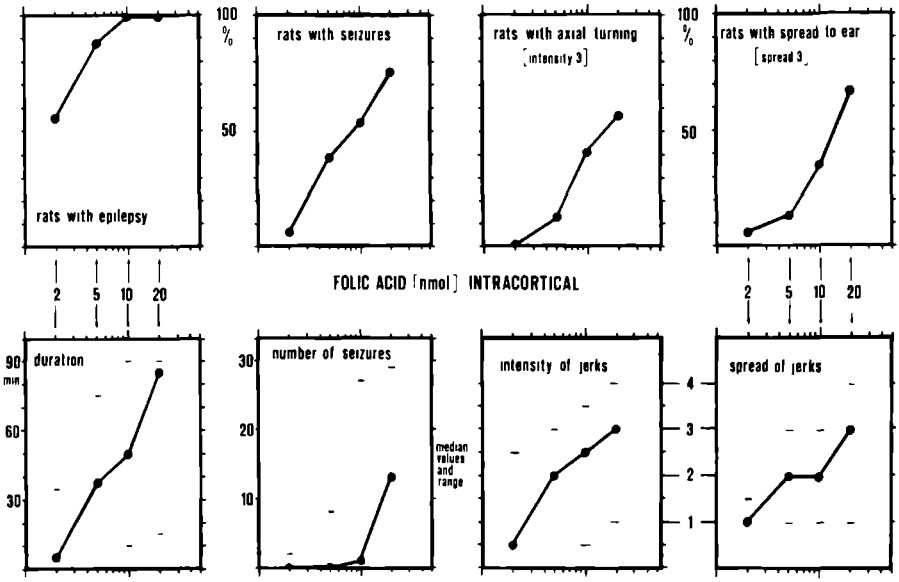


Fig. 1

Fig. 1
Observation of the epilepsy in rats after intracortical injection of different doses of folic acid. Rats were observed for 90 min. The groups consisted of 12 (20 nmol), 17 (10 nmol), 8 (5 nmol) and 16 (2 nmol) animals. The upper panels show percentages, the lower panels show median values. Details of spread and intensity are given in Materials and Methods.

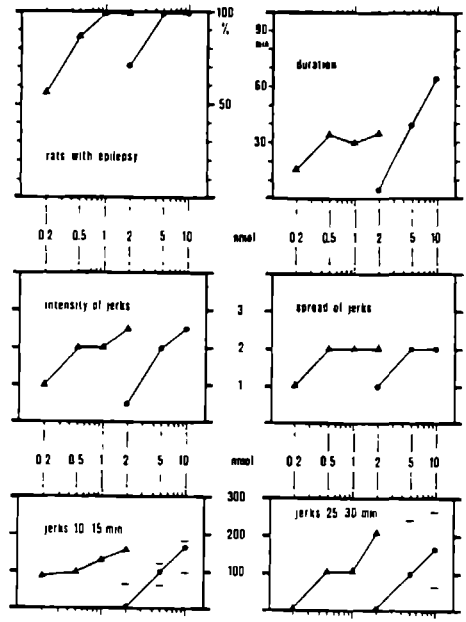


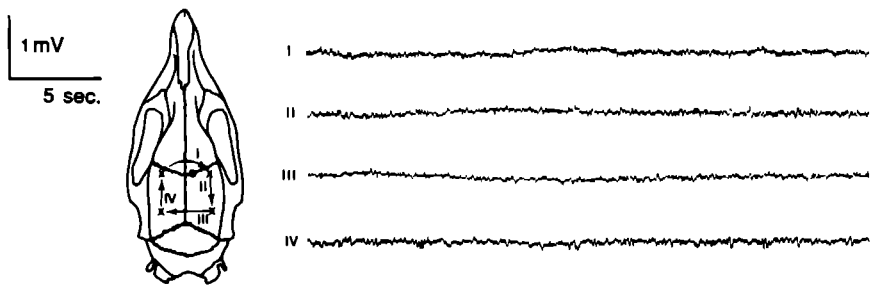
Fig. 3

Fig. 3
Observation of the epilepsy in rats after intracortical injection of different doses of folic acid (circles) and bicuculline methylchloride (triangles). This population was different from that in fig 1. For the duration, spread (= extention) and number of jerks the median values are given (n=7). The range of the jerks for folic acid is given in the lowest panels.

RESULTS 2 : Electrocorticographical effects of the drugs.

Folic acid. (fig. 4)

After intracortical injection of 5 nmol folic acid (10 rats) singular spike-waves appeared on the leads directly adjacent to the injection site within 2 min (fig 4a). The spikes increased in amplitude until after 5-10 min a maximum was reached. From the moment the jerks of the hindlimb were visible, an excellent correlation could be established between the spikes and the independently observed jerks (fig 4b). The jerk frequency was maximal (0.2-0.3 Hz) between 5 and 15 min. One hour after injection no more spike-waves or jerks could be observed. In 3 of the 10 rats 7-11 seizures were observed (fig 4c) while in 4 of the rats only one or two seizures occurred.



***Baseline** ECoG: 1 week after implantation of the cannula and the electrodes. Bipolar recordings: the position of the electrodes and of the cannula is shown. Both olfactory electrodes serve as reference for the amplifier.*

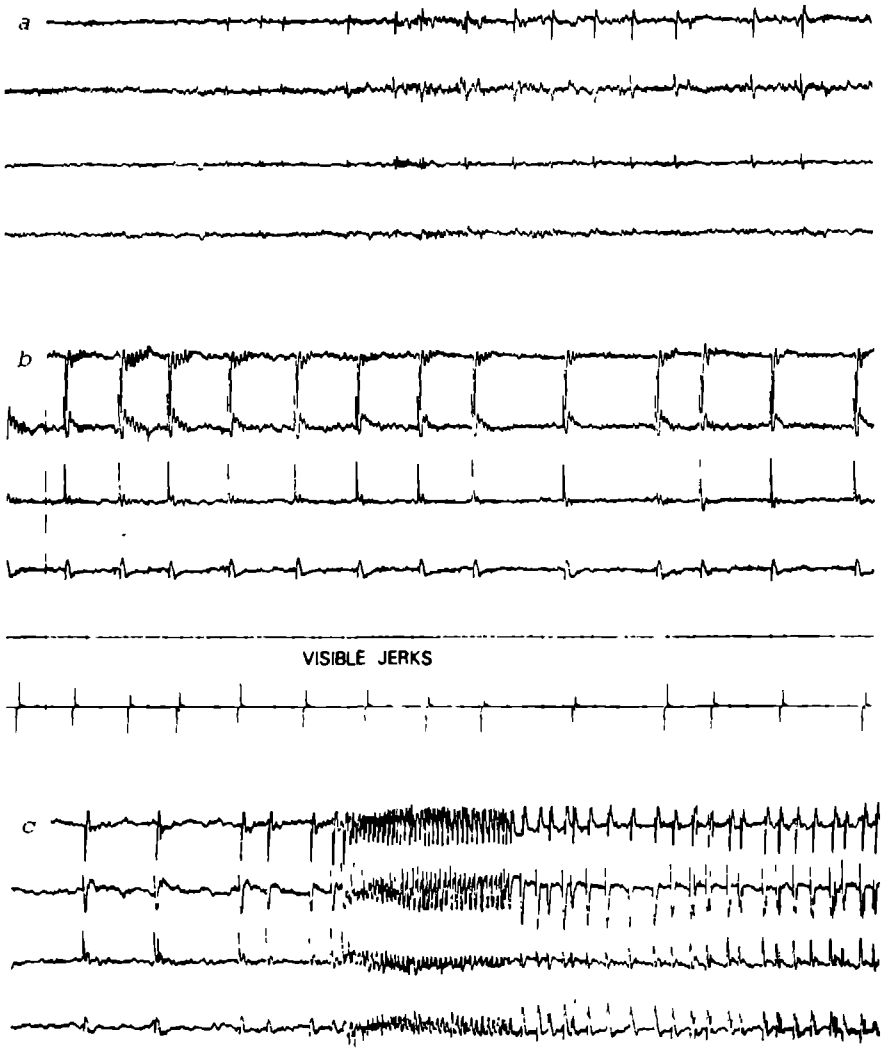


Fig. 4
Electrocorticographical recordings of a rat intracortically injected with 5 nmol ***folic acid.***

a. One minute after injection. First spikes, the jerks are not yet visible and appear one min later

b. Eight min after injection. Every spike coincidence with a clearly visible jerk of the left hindlimb (marked on the lowest line)

c. Thirty tree min after injection, example of a seizure.

Bicuculline. (fig. 5)

Injection of 0.5-2 nmol bicuculline methylchloride (7 rats) produced a similar pattern of electrocorticographic signs. Large spikes were immediately visible after injection and showed an excellent correlation with independently observed jerks (fig 5a,b). The frequency (0.5-0.6 Hz) was higher than that observed for folic acid. Seizures were observed as well.

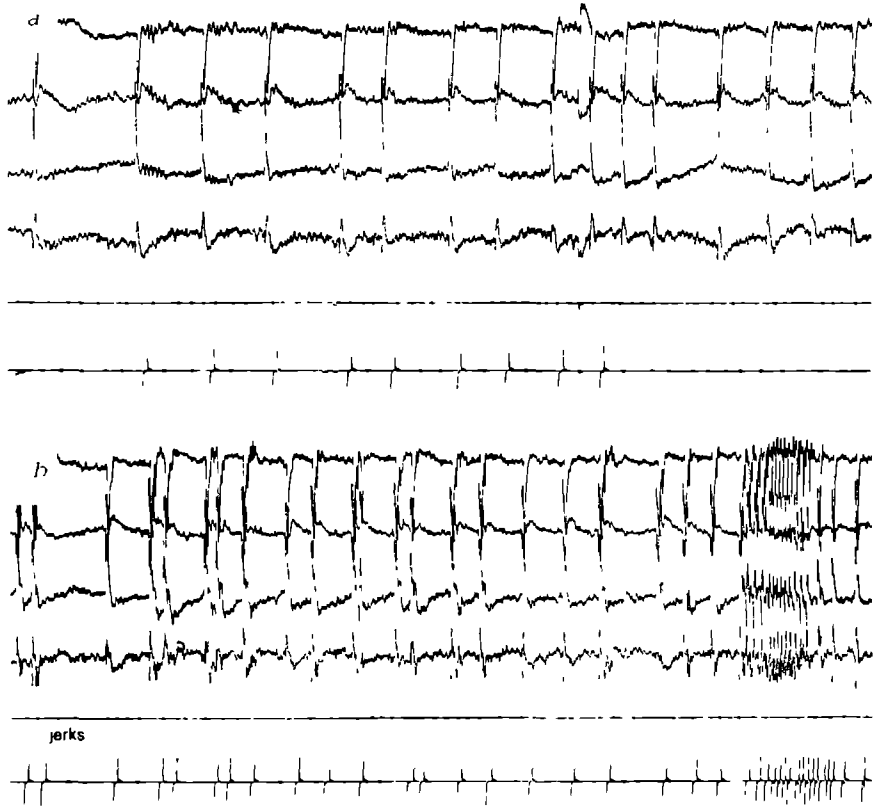


Fig. 5
Electrocorticographical recordings of a rat intracortically injected with 2 nmol ***bicuculline*** *methylchloride.*

a. One min after injection. Spikes correlate with independently observed jerks of the left hindlimb (marked on the lowest line).

b. Eleven min after injection. A seizure is registered.

Penicillin. (fig. 6)

Penicillin (10-1000 I.U., i.e. 17-170 nmol) (2 rats) also produced a similar pattern of spike-waves and seizures. The start resembled that of folic acid in that small spikes rapidly increased in amplitude. The spike frequency was high from the start (0.4-0.5 Hz) and the spike-waves correlated well with observed limb jerks (fig 6a). Only the higher dose (170 nmol) induced seizures (fig 6b).

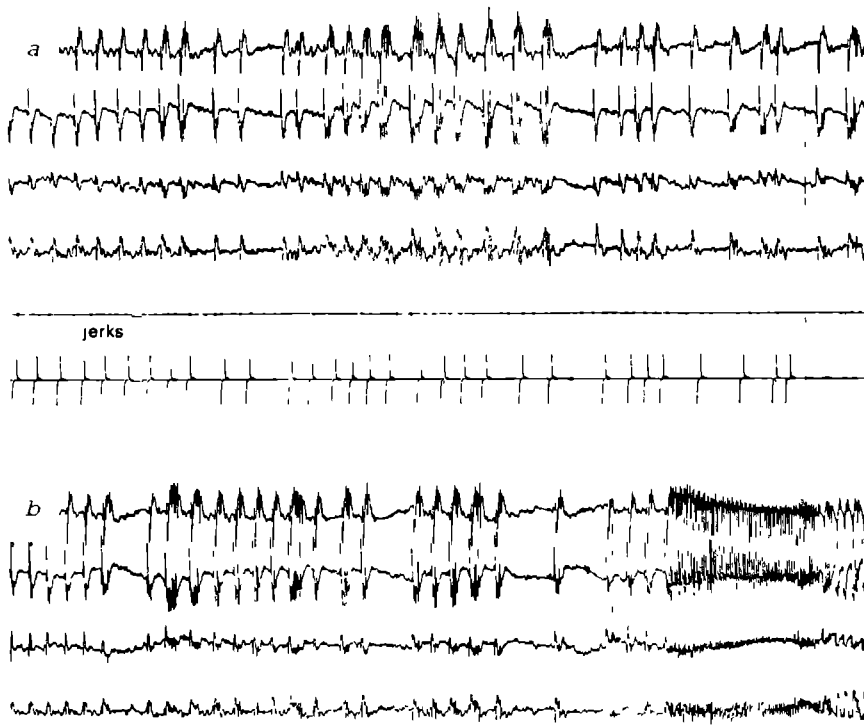


Fig. 6
Electrocorticographical recordings of a rat intracortically injected with 170 nmol ***penicillin.***

a. Forty min after injection. Spikes correlate with independently observed jerks. The jerks spread to the right limbs.

b. A seizure, sixty-nine minutes after injection.

Picrotoxin. (fig. 7)

Application of 2 nmol picrotoxin (n=4) produced singular spike-waves, similar to those described for folic acid and bicuculline and penicillin (fig 7a). About 3 min after the start of the injection spikes appeared which increased in amplitude until a maximum was reached at about 10 min. The spikes were accompanied by myoclonic jerks of the left hindleg. Seizures were observed as well (fig 7b). The duration of phenomena at this 2 nmol dose was about one hour.

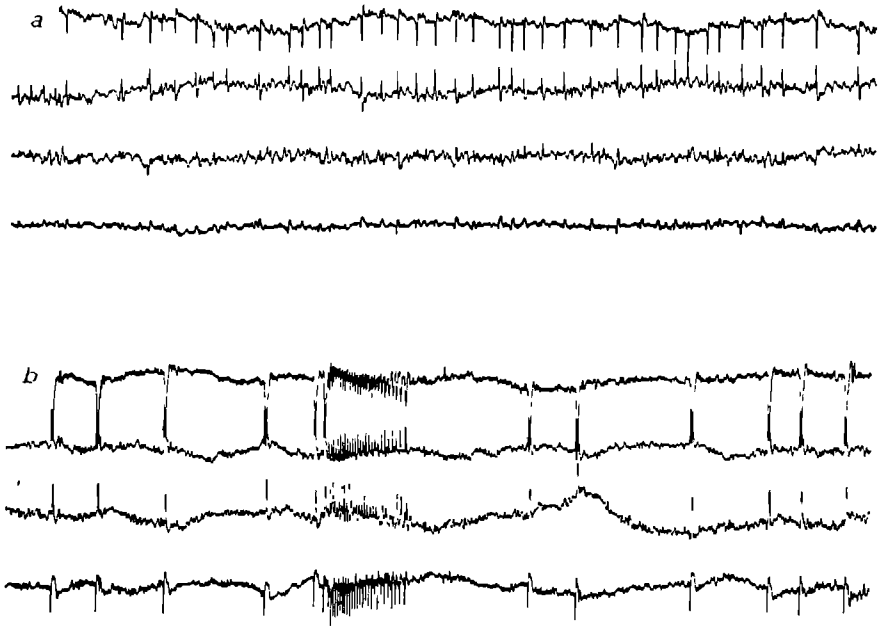


Fig. 7
Electrocorticographical recordings of a rat intracortically injected with 2 nmol ***picrotoxin.***
a. Four min after injection. Spikes appear on the ECG.
b. Spikes (correlating with jerks) and a seizure.

Strychnine. (fig 8)

Within 2 4 min after injection of 100 nmol strychnine (3 rats) spike waves were observed, which almost immediately reached maximal amplitudes (fig 8a) Limb jerks correlating with spikes were observed, but the relation was less strong than obtained with the prior named compounds (fig 8b) The dose did not induce seizures

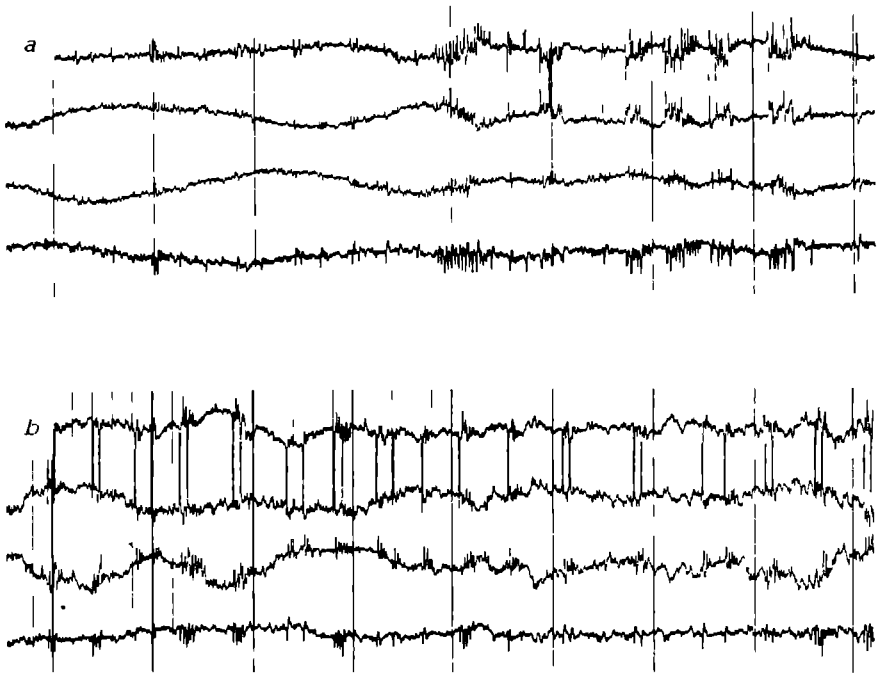


Fig 8
Electrocorticographical recordings of a rat intracortically injected with 100 nmol ***strychnine.***

a *Two min after injection spikes are appearing, weak left hindlimb jerks were observed*

b *Twenty one min after injection Spikes, not correlating with jerks*

Kainic acid. (fig 9)

A totally different pattern of electrocorticographic and behavioral signs was produced by kainic acid (fig 9a) Kainic acid was tested in doses of 1 nmol (4 rats) and 0 3-5 nmol (3 rats) For all doses electrographic signs were first seen between 4 and 30 min after injection Very high frequency spikes with rapidly increasing amplitude appeared and stopped abruptly The spikes complexes disappeared and reappeared in steady progression For the low doses (0 3 and 1 nmol) these events were often not related to any visible motor effects (fig 9b) Limb jerks and seizures related to spike complexes were observed only for the high dose (5nmol), but not always during some of these spike complexes the rats were lying quietly while at other times only the general activity (walking sniffing) was increased In contrast to the results described above for the first four compounds where jerks invariably started from the contralateral hindlimb, the jerks induced by kainic acid were sometimes in the ipsilateral front- or hindleg With time the spike complexes were preceded by repeated single spikes, these spikes were however never accompanied by limbjerks (fig 9c) The duration of the events was dose dependent 0 3 nmol produced effects for 30 min, 1 nmol for 30-90 min, the 5 nmol dose for more than 120 min

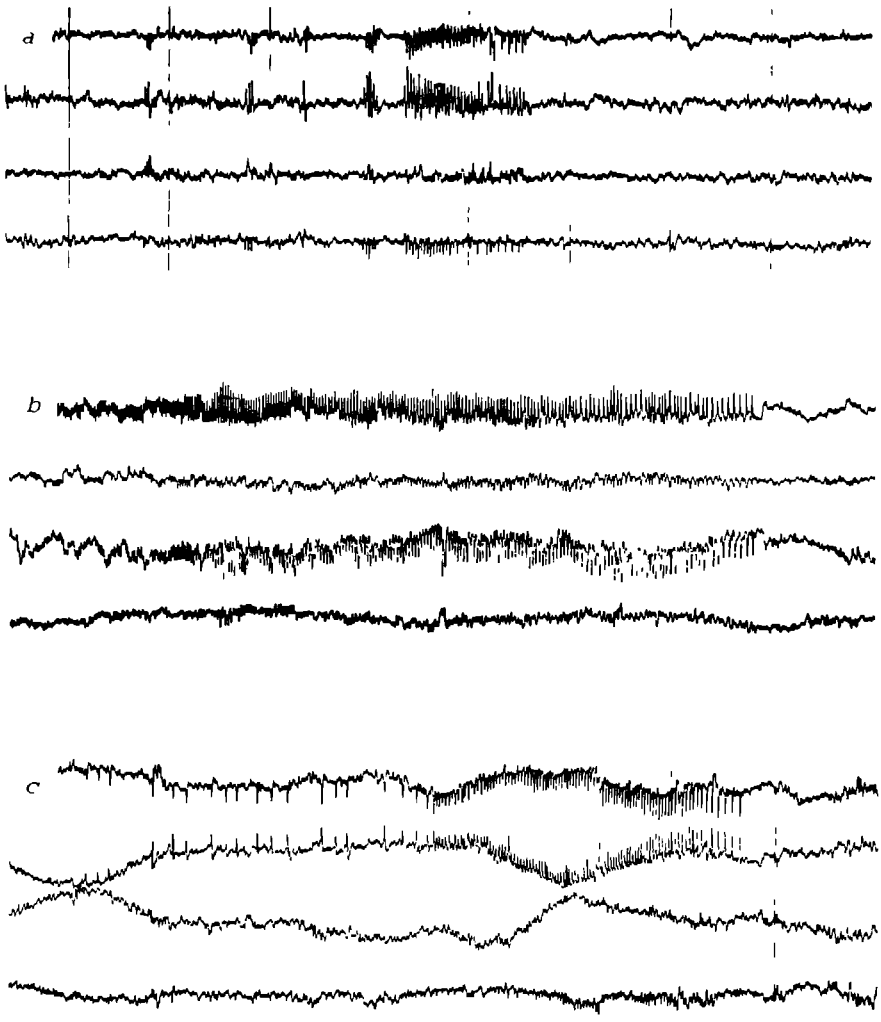


Fig 9
Electrocorticographical recordings of rats intracortically injected with ***kainic acid.***

a. *Thirty-four min after 0.3 nmol. Single spikes and a spike complex without any behavioral or motor effects*
b. *Four min after 1 nmol. A spike complex, no clear behavioral changes were observed.*
c. *Fifty-seven min after 1 nmol. Repetitive single spikes without any sign of behavioral or motor changes are followed by a spike complex.*

Carbachol. (fig. 10)
Low doses (1-2 nmol) produced complexes of high frequency spikes (fig 10a)(4 rats). No jerks were observed. The total duration of the events was about 20 min. Higher doses (5-20 nmol) produced long lasting complexes of high frequency spikes, with different patterns of amplitudes (fig 10b). These doses resulted in generalized tremors. Occasionally observed jerks never could be related to individual spikes.

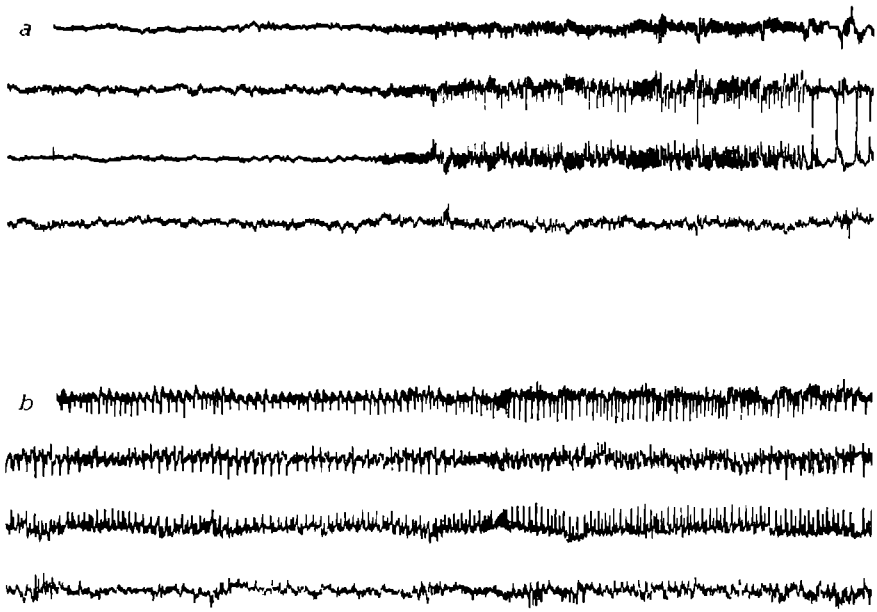


Fig. 10
Electrocorticographical recordings of rats intracortically injected with ***carbachol.***

a. *Four min after injection of 2 nmol. Very high frequency spikes without observable abnormalities.*
b. *Twelve min after 5 nmol. Constant spike formation coincides with the development of generalized tremors.*

Neostigmine. (fig. 11)

Neostigmine, injected in a dose of 100 nmol (2 rats), produced similar effects as did carbachol, but with a different time course. Ten to twenty min after injection spike complexes appeared (fig 11a). On observation the animals showed tremors but no jerks. The complexes appeared throughout the registration period (90 min). In the later stages generalized jerks were sometimes observed but a relation between the spike complexes and the motor events was not found (fig 11b).

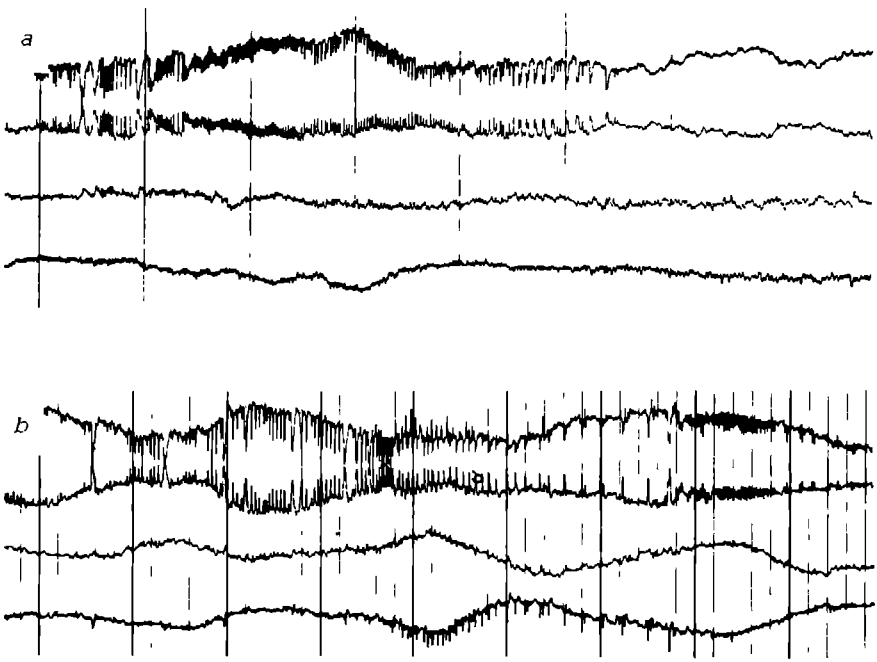


Fig. 11
Electrocorticographical recordings of a rat intracortically injected with 100 nmol ***neostigmine.***

a. Thirty min after injection. spike complex without any related motor effects.
b. Eighty-nine min. some generalized jerks were observed, but not related to individual parts of the complex.

RESULTS 3 . Cortical concentrations of folic acid.

Small blocks of cortical tissue including the cannula tract did not contain folic acid in noninjected or saline injected rats The cortical tissue blocks weighted 20-35 mg and contained 15-30 % of the injected amount, determined between 2 and 20 minutes after injection

A time curve was produced by determining the concentration of folic acid after injection of 5 nmol The results are presented in table 2 While the upper layer showed initially high but in time decreasing levels the the amounts in the middle layer increased in time Assuming that 1 g of tissue is equivalent to 1ml the concentration in the upper and middle layers varied from 10 to 90 µM, and was 35 µM after 20 min in the middle layer where the tip of the cannula was situated

Table 2 Time dependent concentrations of folic acid around the cannula after injection of 5 nmol in the cortex

time Layer	2 min	10 min	20 min
upper	87.8 ± 17.5	81.9 ± 16.3	51.9 ± 4.8
middle (nmol/g)	14.7 ± 6.1	29.0 ± 3.1	34.5 ± 8.4
lower	3.2 ± 0.5	14.5 ± 7.7	16.1 ± 5.9
found (nmol)	1.0 ± 0.1	1.5 ± 0.2	.65 ± .12
jerk intensity	nd	1	1.5

Rats were injected with 5 nmol folic acid, of which 0 65 - 1 5 nmoles was found back Concentrations are means ± SEM, n=5 The top intensity in the 5 min period before decapitation is given as the median value of the 5 animals At the 2 min period no reliable scoring of the intensity was possible

DISCUSSION.

The model.

Folic acid injected into the neocortex produces a clinical syndrome, characterized by myoclonic jerks The jerks are easy to quantify and appear to be dose dependent We have used intraneocortical application of folic acid as an in vivo model to study partial epilepsy This model has some advantages compared to the model described by Arends (who used heat lesions to destroy the blood brain barrier after which he applied the drugs penferal [1]) because a) the technique is very simple, b) the animals can be injected many times, c) from this it follows that the animals can be their own controls and d) the effective dosage of the convulsive or anticonvulsive drugs of study can be defined very reliably

Comparison of the epileptogenic actions.

In order to study the mechanism of the epileptogenic action of folic acid we compared in our model the action of folic acid to that of convulsants with a better known mechanism Clinical and electrographic features have been studied

All tested compounds induced epileptic activity when injected directly into the neocortex, in conformity with earlier reports in which the compounds were tested in other brain regions, or by other techniques [4, 6, 10, 12, 21, 37, 39, 45, 46] Roughly, two groups of convulsive patterns could be distinguished

- The clinical signs of group 1 consisted essentially of myoclonic unilateral jerks of the hindlimb The electrographic signs showed singular spike-waves, which were synchronized with limbjerks The clinical and electrographical signs were easy to quantify Group 1 convulsions are induced by folic acid, bicuculline, picrotoxin, penicillin and strychnine

- The clinical effects of group 2 were essentially characterized by a limbic character Polyspike activity was observed, frequently not synchronized with clinical signs The clinical signs are variable and not easy to quantify Group 2 convulsions are induced by kainic acid, carbachol and neostigmine

Characteristics of the two drug-groups.

- A common biochemical feature of the drugs inducing group 1 effects is that these compounds are thought to interfere with GABA mediated inhibition. Bicuculline is a well known GABA antagonist at the $GABA_A$ receptor complex [18] Picrotoxin is thought to block directly the permeability of the GABA-gated Cl^-

channel [18] Partial GABA antagonism is the supposed mechanism of penicillin [3], though the exact point of interaction is not known [28] Strychnine is a glycine antagonist However strychnine binds with the $GABA_A$ sites as well [8] Moreover a coexistence of GABA and glycine receptors has been suggested [49] The epileptic action of strychnine may results from an blockage of GABA- or glycine mediated inhibition In conclusion, all compounds in group 1 seem to be disinhibitory substances

- The substances in group 2 seem to have direct excitatory potencies themselves Kainic acid is a glutamate agonist [30] Glutamate is an excitatory neurotransmitter Carbachol is an acetylcholine agonist Acetylcholine may have direct excitatory properties in certain brain areas [46] Neostigmine is an acetylcholinesterase inhibitor, thus potentiates the action of acetylcholine [37]

Mechanism of folic acid action.

These in vivo findings suggest that folic acid blocks the inhibitory system rather than that it potentiates the excitatory system This result strengthens the in vitro electrophysiological results published before a blockade of the GABA response was found to be induced by folic acid [38] The molecular mechanism of this action remains to be elucidated

Following intracortical injection of 5 nmoles of folic acid, the concentration in the brain near the cannula is 10 µM - 90 µM, after injection of 30 nmol, 80 µg/g = 180 µM was found This data are in agreement with earlier results from our laboratory the folic acid concentration after intravenous injection (after a heat lesion of the blood brain barrier) has a value of 150 µM in the focus [23]

CONCLUSION.

- The epileptic phenomena induced by folic acid resemble closely those induced by disinhibitory compounds, but differ in many respects from those induced by direct excitatory drugs
- The effective folate concentrations are of micromolar order

These observations were the basis of our further biochemical studies on the influence of folates on the $GABA_A$ receptor complex We refer to chapters 6 and 7 for our reports on this investigations

Epilepsy Res., 1 (1987) 321-327

R(-)-BACLOFEN: FOCAL EPILEPSY AFTER INTRACORTICAL ADMINISTRATION IN THE RAT

C M van Rijn M J van Berlo M G P Feenstra
M L F Schoofs en O R Hommes

SUMMARY

R( ) or S(+)baclofen were injected into lamina IV-V of the sensorimotor cortex of the rat Clinical observation and ECoG registration revealed that partial epilepsy with focal motor symptoms developed following injection of R( )-baclofen with an ED_{50} of 0 25 nmoles a mean latency of 17 min independent of the dose and a duration of more than 5 h at a dose of 5 nmoles S(+)-Baclofen was ineffective at doses of up to 5 nmoles (2 x ED_{100} (-) baclofen) indicating a stereoselective action of the (-)-isomer

INTRODUCTION.

There is at present considerable interest in the role of gamma-aminobutyric acid (GABA) in seizure disorders[23,26] Impaired GABA-mediated inhibition is probably one of the cellular abnormalities leading to focal epilepsy[10] It has been suggested that an important cause of seizures may be the loss of inhibitory GABAergic nerve terminals at sites of focal cortical epilepsy[18] We are studying focal epilepsy by intracortical application of epileptogenic and antiepileptic substances including GABA agonists and antagonists As part of this investigation we studied in our in vivo model the effect of the GABA derivative baclofen β-p-chlorophenyl-γ-aminobutyric acid

Pharmacology.

GABA is an important inhibitory neurotransmitter[10] The receptors for this neurotransmitter can be classified as

- The classical $GABA_A$ receptors located on the postsynaptic membrane, are linked to chloride channels such that receptor activation by GABA leads mostly to

an inward movement of Cl^- resulting in a hyperpolarisation i e in an inhibition of the postsynaptic cell[6] Bicuculline antagonizes this action of GABA whereas muscimol mimics it Baclofen has no effect on these $GABA_A$ receptors[4]

- In 1980 Bowery et al[5] described $GABA_B$ receptors By definition these $GABA_B$ receptors are activated by ( ) baclofen and GABA and are not blocked by bicuculline Muscimol weakly activates these receptors[6] The overall distribution of $GABA_B$ sites differs from that of the $GABA_A$ sites In the cerebral cortex e g there is a high density of $GABA_A$ sites and a lower concentration of $GABA_B$ sites[10]

Activation of $GABA_B$ receptors located on presynaptic excitatory terminals is thought to lead to a reduction of evoked excitatory neurotransmitter release resulting in a decreased excitation of the postsynaptic cell[25 27 29] This effect is probably due to a blocked inward flux of Ca^{2+} and a decreased Ca^{2+}-dependent vesicular release process[6] Indeed many studies have shown an inhibitory action on the firing of neurones in in vivo animal studies[7 9 14 22] Baclofen is used clinically to alleviate spastic disorders (review[4])

In addition to these presynaptic $GABA_B$ receptor effects a postsynaptic action has recently been reported[24] It is suggested that baclofen has a post-synaptic effect by increasing membrane potassium conductance resulting in hyperpolarisation Activation of $GABA_B$ sites located on inhibitory interneurones would then result in disinhibition[24] In addition to the inhibitory action of baclofen some authors report excitatory or disinhibitory actions in in vivo experiments[11 16 20 21] In in vitro experiments inhibitory[1 2 19 28] and disinhibitory[15] or excitatory[24] actions of baclofen were found

As the mechanisms of action of baclofen are not yet clear, predictions of in vivo effects of topical administration of baclofen are difficult to make We report focal epilepsy induced by intracortical administration of R(-)-baclofen in the rat

MATERIALS AND METHODS

Subjects.

The subjects were male Wistar albino rats with a weight of 200 + 10 g at time of surgery for those receiving a cannula only, and 260 ± 10 g for those receiving

electrodes as well The animals were individually housed and allowed access to food and water ad libitum A 12 h light, 12 h dark cycle was maintained, light on at 7 a m The experiments took place in the light phase

Surgery.

Details of the methods of application will be published elsewhere (Feenstra et al, in prep) In short

The animals were anaesthetized by pentobarbital A polyethylene cannula (outer diameter 0 8 mm, inner diameter 0 4 mm) was implanted through a drill hole in the skull 1 4 mm to the right of bregma, where the sensorimotor cortex of the left hind leg is situated[12] The cannula was fixed by acrylic cement The tip of the cannula was 2 mm beneath the upper surface of the skull histological examination revealed that the tip was in lamina IV or V of the cortex Cortex laminae were determined according to Krieg[13] The cannula could be connected to a flexible injection system This permitted free movement during administration of the drugs

Some animals received 4 epidural (and 2 nasal reference) electrodes on the skull as well, positions related to the bregma anterior 0 0 mm, lateral 3 6 mm, posterior 6 0 mm, lateral 4 0 mm (references anterior 6 0 mm, lateral 1 5 mm) The electrodes, stainless steel screws 1 mm x 2 mm, were connected to a minisocket (MTA, Canon ITT) and embedded in acrylic cement Free movement remained possible during ECoG registration The animals were left to recover from surgery for 5-7 days

The experiments.

The drugs, dissolved in water, were administered into the cortex through the cannula in a volume of 0 5 µl For doses exceeding 10 nmoles a larger volume was injected, maximally 2 µl The injections were performed with a velocity of 0 5 µl/min Each dose was tested on all the animals in the group, only 1 dose/day

The ECoGs were recorded on a Siemens Elema 8-channel mingograph The amplification filter had an upper limit of 15 Hz and a time constant of 1 2 sec

We tested (-)-baclofen and (+)-baclofen as follows.

Animals with a cannula only 14 animals received (-)-baclofen in a dose varying from 0 05 to 10 nmoles, 4 animals received (+)-baclofen in a dose varying from

0 05 to 10 nmoles, 9 animals received (+)-baclofen in a dose varying from 10 to 40 nmoles In the past we studied the effect of NaCl solution extensively
Animals carrying electrodes as well were treated as follows
1 animal received (-)-baclofen in a dose varying from 0 05 to 10 nmoles, 1 animal received (+)-baclofen in a dose from 0 05 to 10 nmoles, 1 animal received (+)-baclofen in a dose up to 80 nmoles in 2 µl, 1 animal received a daily dose of 0 05 nmoles (-)-baclofen, for 15 days, 1 animal received NaCl in the same concentrations as baclofen
The animals were observed for 1 5 h following injection If an animal showed abnormal behaviour the delay time was noted and the abnormalities were described, no quantifications were made
Each ECoG registration was started at least 0 5 h in advance of injection of the drug to have a sufficient duration of baseline registration The events in the animals carrying electrodes were marked on the registration paper For the dose of 5 nmoles (-)-baclofen the registration and observation were extended to 8 h

Drugs.
R(-)- and S(+)-baclofen HCl were kindly donated by Ciba-Geigy BV, Arnhem, The Netherlands

RESULTS.

Following injection of (-)-baclofen into the right sensorimotor cortex of the rat, hind leg area, lamina IV-V, clinical as well as ECoG abnormalities developed
The clinical events were characterized by intermittent myoclonic jerks of the hind leg The jerks were clearly observable saccadic flexion movements of the leg, of a constant pattern They resembled focal epileptic phenomena induced in this model by folic acid, penicillin or bicuculline The myoclonus did not spread to other parts of the body, nor did generalized seizures develop Apart from one animal, who grew aggressive once only, no other behavioural abnormalities were observed consciousness was unimpaired, grooming behaviour was normal We never saw abnormalities following injection of NaCl

The clinical symptoms were accompanied by spike-wave complexes and solitary spikes on the ECoG (Fig 1$_{1-9}$) The complexes, which had a duration of about 1 sec consisted of spike-waves in a frequency of 6/sec (Fig 1$_4$) There were 10 complexes in 1 min at the most The voltage of the spikes was about 500 µV (Fig 1$_{3,4}$) The spike-wave complexes as well as the spikes correlated with visible jerks, but some jerks had no correlates on the ECoG (Fig 1$_6$) On the control ECoGs registered after injection of NaCl isomolar to the tested dose of baclofen no discharges were seen (Fig 1$_1$)

Of the clinically observed animals 50% (7/14) showed this response at a dose of 0 25 nmoles (-)-baclofen All the animals responded to 2 5 nmoles (-)-baclofen (ED_{100}) (Fig 2) No discharges were seen on the ECoG after 15 times a daily dose of 0 05 nmoles (-)-baclofen (Fig 1$_2$) (+)-Baclofen had no influence on the behaviour and the ECoG up to a dose of 5 nmoles (2 x ED_{100} for (-)-baclofen) In the observation group 4 out of 9 animals showed very weak jerks following 40 nmoles (+)-baclofen (dissolved in 2 µl) The animal carrying electrodes responded only to 80 nmoles (+)-baclofen, on the ECoG spike-wave complexes not resembling those described above were seen (Fig 1$_5$) The weak jerks corresponded with these ECoG abnormalities

The jerks following (-)-baclofen as well as those following (+)-baclofen started with a median delay of 16 min, range 3-36 min (mean 17 ± 7 min) No correlation between the dose and the delay time could be seen

The duration of the clinical signs was 5 h in the animal we observed and registered for 8 h following 5 nmoles (-)-baclofen Spindle-like abnormalities on the ECoG were still present after 8 h, but had disappeared after 24 h (Fig 1$_{7,8,9}$)

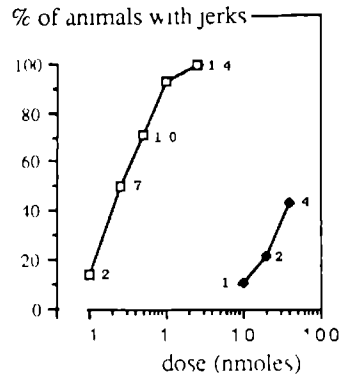


Fig 2 *(Fig 1 see next page)*

Dose-response curve
Intracortically injected dose of (-)-baclofen (n = 14) (-□-) and of (+)-baclofen (n = 9) (-●-) needed to produce visible jerks of the hind leg The jerks were clearly visible, observed during at least 10 min

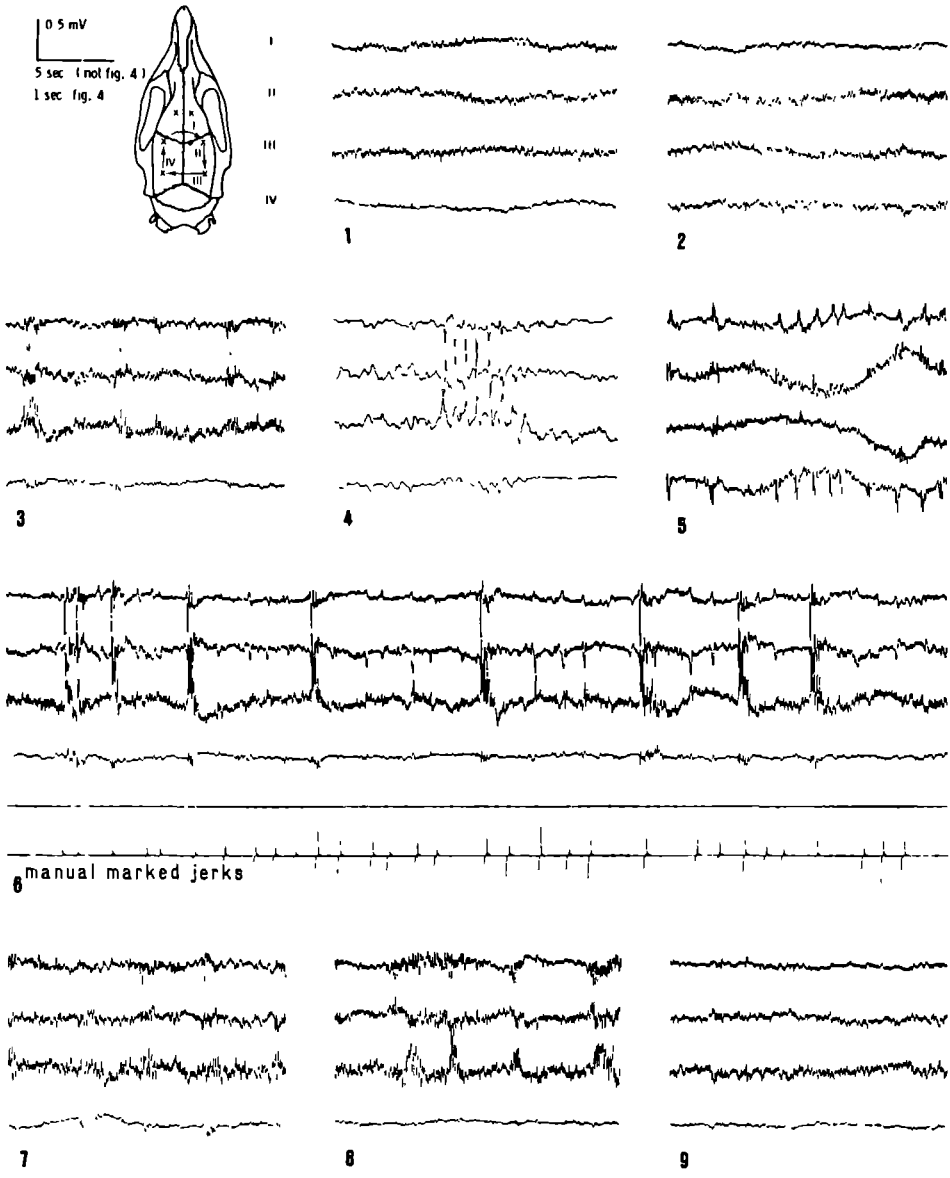


Fig. 1
ECoGs: bipolar recordings; the position of the electrodes and the cannula is shown. Both olfactory electrodes serve as reference for the amplifier.
1, baseline: 1 week after implantation of the cannula and the electrodes;
2, 40 min after administration of 0.05 nmole (-)-baclofen on the 8th day: no abnormalities;

3. *40 min after administration of 2 5 nmoles (-)-baclofen spike-wave complexes with a duration of about 1 sec,*
4. *as 3, fast paper speed a complex consisting of spike-waves with a frequency of 6/sec,*
5. *40 min after administration of 80 nmoles (+)-baclofen spike-wave complexes not resembling those in 3,*
6. *40 min after administration of 5 nmoles (-)-baclofen spike-waves and spikes correlating with visible jerks, some jerks have no reflection on the ECoG,*
7. *2 5 h after 5 nmoles (-)-baclofen spike wave complexes are still present, jerks were seen,*
8. *7 5 h after 5 nmoles (-)-baclofen spindle-like abnormalities, no visible jerks anymore,*
9. *24 h after 5 nmoles (-)-baclofen no abnormalities on the ECoG*

DISCUSSION.

Our results show that intracortical administration of baclofen in the rat causes partial epilepsy with focal motor symptoms The action shows stereoselectivity for the (-)-isomer

The ED_{100} is 2 5 nmoles This dose is comparable to that of bicuculline-methochloride or folic acid using the same model (Chapter 2) Also the appearance of the jerks, viz , flexion movements of the hind leg, is comparable to that observed following the administration of bicuculline or folic acid However some important differences were noted between baclofen, bicuculline and folic acid the saccadic feature of the flexion is specific for baclofen The jerks following bicuculline or folic acid occur singly (Chapter 2) The clinical signs induced by baclofen are limited to movements of the hind leg, we never observed any spreading to other parts of the body, nor were generalized seizures seen, not even after injecting 10 nmoles (-)-baclofen Generalized seizures do occur following bicuculline or folic acid In addition the time-course of the effect shows a different pattern the onset of the effect of bicuculline and folic acid is rapid, always within a few minutes, and the duration of the effect at the doses mentioned here is less than 1 5 h In contrast, we observed a considerable delay of 17 min and a duration of several hours in the effect of (-)-baclofen Tested in our model up to 100 nmoles, muscimol, a potent $GABA_A$ and weak $GABA_B$ activator, does not induce epileptic phenomena

Some earlier reports on the possible epileptogenic action of baclofen have been published in the baboon epileptic responses to photic stimulation could be abolished by 2 mg/kg i v (-) baclofen (10^{-5} moles), "but a toxic syndrome characterized by impaired alertness, ataxia, loss of muscle tone, rhythmic limb jerks and abnormal slowing of the background EEG rhythms with irregular or rhythmic spikes and spikes and waves" occurred following 4-8 mg/kg i v [20] In mice 100 mg/kg i p baclofen causes generalized convulsions[17], as it does in rats after 50-100 mg/kg p o [11]

In contrast to the epileptogenic action, some authors do find antiepileptogenic properties of baclofen baclofen affords protection against convulsions induced by electroshocks, hyperbaric oxygen, 3-mercaptopropionic acid and audiogenic stimuli in mice, but not against convulsions induced by pentetrazole strychnine, or picrotoxine (ref 3 10 mg/kg i p , ref 8 15 mg/kg i p , ref 17 3 mg/kg p o)

The results of Meldrum[20] suggest a biphasic action of (-)-baclofen, being inhibitory in low and epileptogenic in high doses We intend to test the antiepileptic action of low dose baclofen in our model

The mechanism of action of baclofen is still unclear Peet and McLennan[24] proposed that activation of postsynaptic $GABA_B$ receptors, located on inhibitory feed-forward neurones, reduces the inhibitory postsynaptic potentials, resulting in a disinhibition Although in our hands the effects of (-)-baclofen show a superficial resemblance to the effects of a disinhibitory substance like bicuculline, the different appearance of both clinical and ECoG signs makes it unlikely that the proposed disinhibition by (-)-baclofen resembles the disinhibition caused by bicuculline

CONCLUSION.

Intracortical application of the GABA derivative R(-)-baclofen produces focal motor epilepsy in rats This action is stereoselective for the (-)-isomer

Acknowledgements.

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A LOW DOSE OF FOLIC ACID IN THE PREPIRIFORM CORTEX OF THE RAT DOES NOT INDUCE EPILEPSY

With the participation of R H J Arts,
J F Rodrigues de Miranda and O R Hommes

SUMMARY.
The amount of folic acid (5 nmoles) needed to induce in all the tested animals epileptic phenomena in the neocortex appears to be ineffective in the prepiriform cortex In contrast bicuculline is more effective in the prepiriform cortex than in the neocortex These observations elicit some questions with respect to the mechanism of action of both folic acid and bicuculline

INTRODUCTION.

Recently, Piredda et al [8] located a small site in the prepiriform cortex of the rat from which bilateral motor seizures can be elicited by a single unilateral injection of extremely low doses of convulsant compounds, such as kainic acid, bicuculline and carbachol [1, 6, 7] They named this site the 'area tempestas' [2] Viewed in the light of our previous studies into the epileptogenicity of folic acid in the neocortex [chapter 2], we considered the possibility that this compound can elicit seizures from the area tempestas as well We evaluated by means of clinical and electrocorticographical observations the effects of injection of folic acid, bicuculline and kainic acid into the area tempestas

MATERIALS AND METHODS.

Subjects and Surgery.

Details of the methods of application are described elsewhere (chapter 2) Some modifications Anaesthetized male Wistar Albino rats (250 - 300 g) were placed in a stereotactic apparatus with the incisor bar at the level of the interaural line A 22 gauge (diameter is 1 6 mm) stainless steel guide cannula was implanted through a drill hole in the skull position related to the bregma 2 4 mm anterior, 3 0 mm to the right The tip of the cannula was 6 5 mm beneath the upper surface of the skull histological examination revealed that the tip was in the prepiriform cortex The stainless steel cannula could be connected to a flexible injection system to permit free movement during administration of the drugs The animals received electrodes on the skull as described in chapter 2 and were left to recover from surgery for 5-7 days

Administration of the drugs.

The drugs dissolved in water, were administered into the prepiriform cortex in a volume of 0 5 µl in two minutes We tested the drugs as follows Before injecting the test drugs, 0 5 µl of a physiological salt solution was injected and the ECoG registered for thirty minutes to have a baseline registration Six animals were injected with 5 nmol folic acid Twenty four hours later 4 of the animals received 0 25 nmol kainic acid and 2 of the animals received 0 10 nmol bicuculline methyl-chloride On the third day the animals were reinjected with 5 nmol folic acid The animals were observed and the ECoG was registered for 90 minutes

RESULTS.

Effects of the drugs.

- **Folic acid** injected on the first day did not induce any behavioral or electrographical abnormalities Reinjection of folic acid on the third day did not induce abnormalities either, while injection with kainic acid on the second day and reinjection on the fourth day did induce limbic seizures

- **Kainic acid:** Within 4 - 30 minutes from the beginning of the administration of kainic acid, epileptic seizures were observed The start of the seizures was characterized by myoclonic jerks of the forelimb contralateral to the injected site progressing to clonic contraction of both forelimbs Subsequently recurrent clinical signs of limbic seizures were observed [5, 10], these included wet dog shakes stereotyped motor activities such as turning round and round anticlockwise, headbobbing, chewing with salivation falling, rearing and headweaving The electrocorticographical signs consisted of mainly ipsilateral, spiking activity with variable frequency (fig 1c) The spike complexes disappeared and reappeared numerous times

- **Bicuculline:** A totally different pattern of electrocorticographic and behavioral signs was produced by bicuculline We observed only myoclonic jerks of the left forelimb accompanied by a turning of the head to the right within 2 min after the start of the injection No limbic signs were observed The ECoG signs consisted of singular spike wave discharges, correlating with the myoclonic jerks The ipsilateral leads showed the highest amplitudes (fig 1b)

Comparison: Prepiriform-cortex versus Neo-cortex.

In the neocortex 5 nmol of folic acid induced in all the tested animals epileptic phenomena This dose did not induce any epileptic signs when injected in the prepiriform cortex This is in contrast to the observations of the effective doses of bicuculline and of kainic acid in the two areas Moreover, the epileptic phenomena induced by the indicated doses of the latter two compounds in the prepiriform cortex, were far more severe than those elicited by these low doses in the neocortex (see chapter 2 and table 1)

Table 1 Epileptogenic effect of convulsants injected in the prepiriform cortex and the neocortex (Data for the neocortex are from chapter 2)

Site	Prepiriform Cortex		Neocortex	
Compound	dose (nmol)	incidence (effect/total)	dose (nmol)	incidence (effect/total)
Folic acid	5	0/6	5	10/10
Kainic acid	0.25	4/4	0.3	3/3
Bicuculline	0.1	2/2	0.5	7/7

Above results are in agreement with those described by Gale and Piredda [2, 6] However some differences with their reports are observed

The electrocorticographical changes in our experiments were mainly recorded from the ipsilateral cortex near the focus, whereas generalized seizures are described by these named authors

- We found clinical differences between the seizures elicited by bicuculline (myoclonic jerks) and those elicited by kainic acid (full limbic seizures), as well as electrographical differences, Gale and Piredda found no clinical, nor ECoG differences between the substances

Possibly we did not inject the compounds into precisely the 'area tempestas' which is only a small part of the prepiriform cortex the area is very small and can be easily missed [6] Histological examination revealed that we did inject into prepiriform cortex

We conclude therefore that the prepiriform cortex has a low susceptibility for folic acid with respect to induce epileptic phenomena, but a high susceptibility for bicuculline In the neocortex, the epileptogenic potency of folic acid is only slightly less than that of bicuculline

DISCUSSION.

In this thesis we hypothesize a relationship between the effects of folic acid and those of bicuculline We propose that a GABAergic inhibitory action may be the biochemical basis of the epileptic phenomena by folic acid However the observed differences between the effects of folic acid and bicuculline as reported in this chapter do evoke some questions

1) Do the observed phenomena induced by either folic acid or bicuculline indeed follow from an interaction of the drugs with the $GABA_A$ complex?
2) If so, do the substances compete for the same binding sites on the complex?

Ad 1) It is reported that besides GABA other bicuculline sensitive neuronal inhibitors are found in the central nervous system [3] So the phenomena induced by bicuculline in the prepiriform cortex need not result from a $GABA_A$ receptor antagonism

On the other hand: there may be regional differences in the sensitivity of receptors to ligands [9]. Recently it was suggested that there are regional differences in the gene expression of subunits of the $GABA_A$ receptor complex [4]. So, our hypothesis (chapter 2) that folic acid interacts with the $GABA_A$ complex need not to be in conflict with the possibility that the phenomena induced by bicuculline do result from an interaction with the same complex as regional differences for the interaction with folic acid may exist.

Ad 2) In the second part of this thesis experiments concerning this question will be described. Therefore we will return to this point in the conclusion.

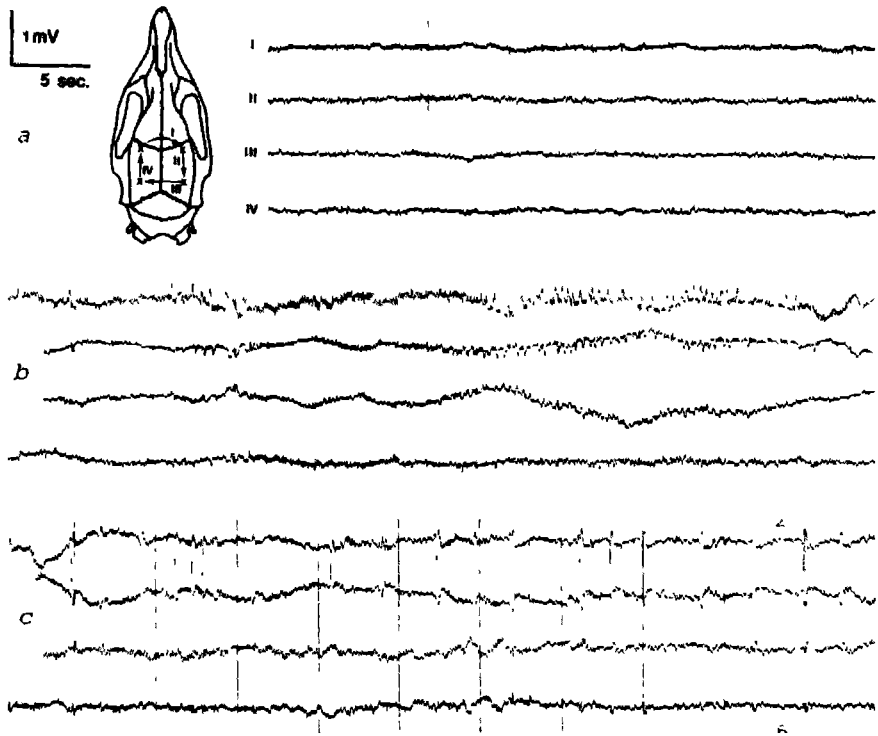

Fig. 1

a ***Baseline*** *ECoG. Bipolar recordings, the position of the electrodes is shown.*

b *Recordings of a rat injected with 0.25 nmol* ***kainic acid*** *into the prepiriform cortex. A spike-wave complex not resembling the single spikes-waves observed in fig 1c is seen.*

c *Recordings of a rat injected with 0.1 nmol* ***bicuculline***-*methylchloride into the prepiriform cortex. Focal single spike-waves are registered.*

In the first part of this thesis we described the in vivo investigations of the epileptogenic mechanism of folic acid

- *Injected into the neocortex folic acid induces epileptic phenomena resembling those induced by GABAergic inhibitory compounds, especially bicuculline and picrotoxin (When injected into the prepiriform cortex however, folic acid does not mimic the action of bicuculline In the conclusion we will return to this last observation)*
- *Major differences are found between the phenomena caused by folic acid and those caused by direct excitatory drugs such as kainic acid*
- *The phenomena induced by the $GABA_B$ agonist baclofen differ from those by folic acid*

From these results, supported by data published before (cf Introduction), we hypothesized that folic acid exerts its epileptogenic action through an interaction with the $GABA_A$ receptor complex

In the second part of the thesis we describe the in vitro tests concerning our hypothesis

In het eerste deel van dit proefschrift werden de in vivo studies beschreven naar het biochemisch mechanisme dat aan de epileptogene werking van foliumzuur ten grondslag ligt

Na injectie in de neocortex veroorzaakt foliumzuur epileptogene verschijnselen die sterk lijken op verschijnselen geinduceerd door GABA-inhiberende stoffen zoals bicuculline en picrotoxine (Echter geinjecteerd in de prepiriforme cortex inviteert foliumzuur het effect van bicuculline niet Op deze waarneming zal in de conclusie nader worden ingegaan)

- *Er worden grote verschillen gezien tussen die verschijnselen die door foliumzuur veroorzaakt worden en die welke het gevolg zijn van direct exciterende stoffen zoals kainezuur*
- *De verschijnselen geinduceerd door de $GABA_B$ agonist baclofen verschillen van die veroorzaakt door foliumzuur*

Naar aanleiding van deze resultaten, gesteund door eerder in de literatuur verschenen gegevens (zie 'Introduction'), stelden we de hypothese op dat foliumzuur epileptogeen werkt doordat het een invloed heeft op het $GABA_A$ receptor complex

In het tweede deel van dit proefschrift worden de in vitro experimenten beschreven die we uitvoerden om deze hypothese te testen

THE BINDING OF THE CAGE CONVULSANT [^{3}H]TBOB TO SITES LINKED TO THE GABA$_A$ RECEPTOR COMPLEX

C M van Rijn, J F Rodrigues de Miranda
T J A M van der Velden O R Hommes

SUMMARY.
[^{3}H]TBOB binds to specific sites on crude synaptic rat brain membranes The dissociation constant K_d determined from saturation experiments is near 8 nM and the receptor density R_T is about 20 pmoles/g wet tissue Non-specific binding constitutes about 35 % of total binding at 4 nM [^{3}H]TBOB
Association of [^{3}H]TBOB is monophasic, but its dissociation is biphasic From the kinetic data K_d values of 8 nM (70 % of the binding sites) and 20 nM (30 % of the binding sites) are estimated These values differ from those previously reported
Specifically bound [^{3}H]TBOB is displaced by picrotoxin and by TBPS A simple competitive interaction of picrotoxin with [^{3}H]TBOB binding is not found Micromolar quantities of the GABAergic facilitating compounds GABA, muscimol, diazepam and pentobarbital, inhibit [^{3}H]TBOB binding in an allosteric manner

INTRODUCTION.

The GABA$_A$-receptor-complex is an oligomeric membrane protein with allosteric binding sites (Enna and Karbon, 1986, Stephenson, 1987) Ligand binding assays have demonstrated at least three distinct recognition sites at the GABA$_A$ receptor complex i e a GABA receptor site, a benzodiazepine site and a picrotoxinin or convulsant site (Bowery et al 1984, Olsen, 1981, Maksay and Simonyi, 1986) A fourth binding site, a barbiturate recognition site, has been postulated (Trifiletti et al , 1985)

The picrotoxinin or convulsive site on the GABA$_A$ complex can be characterized with [^{3}H]-α-dihydropicrotoxinin ([^{3}H]DHP) (Ticku et al , 1978) and with [^{35}S]-t-butylbicylophosphorothionate ([^{35}S]TBPS, Maksay and Simonyi, 1986, Ramanjaneyulu and Ticku, 1984, Squires et al , 1983, Wong et al , 1984) A disad-

vantage of using [^{3}H]DHP is its low affinity (K_d = 1-2 µM) and consequently its high non-specific binding. A handicap of [^{35}S]TBPS (K_d = 17-25 nM) is the short half life of the radiolabel. Recently a new tritium labelled probe was introduced [^{3}H]-t-butylbicycloorthobenzoate ([^{3}H]TBOB) with a reported K_d of 60 nM (Lawrence et al., 1985). TBOB is structurally related to TBPS.

As part of our study on the influence of convulsive compounds on the $GABA_A$ receptor complex, we determined the binding characteristics of [^{3}H]TBOB and the interaction of the binding with a) GABAergic facilitating agents that produce anti-convulsant and sedative effects in vivo, and b) with GABAergic inhibitory agents. Only a few reports using [^{3}H]TBOB have been published (Lawrence et al. 1985; O'Conner and McEwen, 1986; Fishman and Gianutsos 1987, Malminen and Korpi 1988; Schwartz and Mindlin 1988). Only one report concerned the binding characteristics of [^{3}H]TBOB (Lawrence et al. 1985). In contrast to the data reported, we observed a high affinity of [^{3}H]TBOB (K_d - 8 nM), and a complex interaction of GABAergic drugs with [^{3}H]TBOB binding.

MATERIALS AND METHODS.

Preparation of the Membranes.

A crude membrane fraction was prepared as described by Lawrence et al. (1985). Male Wistar rats (200 ± 20 g) were killed by decapitation. The whole brain was weighed and homogenized in 9 volumes 0.32 M sucrose at 0 °C with a Teflon-glass homogenizer. The homogenate was centrifuged at 1000 g for 10 min at 4 °C; the supernatant was decanted and centrifuged at 9000 g for 20 min at 4 °C. The pellet was suspended in 50 mM sodium-potassium-phosphate buffer, pH 7.4 containing 500 mM NaCl (assay buffer) and centrifuged at 16000 g for 10 min at 4 °C. Protein concentration in the pellet was quantified by the method of Lowry et al. (1951) and was approximately 4 % of wet tissue weight.

Receptor Assays: General Procedure.

Membrane pellets were resuspended in assay buffer. Glass tubes (5 ml) received consecutively 100 µl of [^{3}H]TBOB (specific activity 54 Ci/mmol; Amersham), 100 µl of buffer with or without competing ligand and 800 µl of tissue

homogenate (25 mg tissue) Incubations were performed at 25 °C usually lasting 45 min for saturation assays and 30 min for inhibition assays Under these incubation conditions the stability of the compound was demonstrated by Scott et al (1987) Incubations were terminated by filtration of 0 8 ml of the incubation mixture through Whatman GF/B filters on a Millipore 12 sample manifold The filters were washed two times with 5 ml ice cold buffer Radioactivity retained in the filters was counted by liquid scintillation spectrometry Specific [^{3}H]TBOB binding was defined as the difference between binding in the absence and presence of 10 µM picrotoxin or 10 µM TBPS and was 60-70 % of total binding at 4 nM [^{3}H]TBOB Experimental data were analyzed and binding parameters calculated by a computer assisted non linear least square curve fitting routine The kinetic data were analyzed by linear least square curve fitting methods All data points are means of duplicates The methodology of analyzing the binding data is described by Bennett and Yamamura (1985) from whom we adopted the following methods

Saturation Binding Assays.

The concentrations of [^{3}H]TBOB ranged from 0 1 nM to 30 nM, in four experiments the concentration reached 60 nM

Saturation binding assays for [^{3}H]TBOB binding were performed in the absence and presence of the tested inhibitors

Kinetic Studies.

For the kinetic studies fixed concentrations of 3H-TBOB were used 3 4 nM or 6 4 nM

For association, incubations were terminated after various time intervals following addition of [^{3}H]TBOB To determine the k_{+1}, association was plotted according to the pseudo first order equation

$$\ln \{B_{eq} / (B_{eq} - B_t)\} = (k_{+1}L + k_{-1})\, t \quad (1)$$

in which B_{eq} and B_t are the amount of [^{3}H]TBOB-receptor complex in equilibrium and at time t during incubation The k_{-1} was determined from dissociation experiments. L is the free ligand concentration and was approximately equal to the added concentration [^{3}H]TBOB since at most 5 % of the [^{3}H]TBOB added was bound

The half-life of association of second-order reactions is given by the following equation

$$t_{½}\ (ass) = \ln 2 / (k_{+1}L + k_{-1}) \quad (2)$$

For dissociation, membrane homogenates were preincubated with a fixed concentration of [^{3}H]TBOB (3 4 nM or 6 4 nM) at 25 °C for 30 min Dissociation was initiated by 10 µM picrotoxin or 10 µM TBPS (time 0) [^{3}H]TBOB binding was determined at various points of time and plotted according to

$$\ln (B_t / B_{eq}) = - k_{-1}t \quad (3)$$

to yield the dissociation rate constant k_{-1} and the half-life of dissociation

$$t_{½}\ (diss) = \ln 2 / k_{-1} \quad (4)$$

The dissociation constant K_d of [^{3}H]TBOB was determined by

$$K_d = k_{-1} / k_{+1} \quad (5)$$

Inhibition Studies.

For inhibition studies a fixed concentration of [^{3}H]TBOB of approximately 4 nM was used We tested the following inhibitors a) the GABAergic facilitating ligands GABA, the GABA agonist muscimol, and diazepam ,a GABA positive ligand for benzodiazepine binding sites and b) the GABAergic inhibitory agents the GABA antagonist bicuculline, ethyl-β-carboline-3-carboxylate (βCCE, a partially negative ligand for benzodiazepine binding sites) and the convulsants picrotoxin and TBPS

The binding affinity constants of the unlabelled ligands were estimated according to the following equation (Cheng and Prusoff,1973)

$$K_I = IC_{50} / (1 + L / K_d) \quad (6)$$

The unlabelled chemicals were obtained from Sigma except TBPS, which was obtained from NEN

RESULTS.

Binding Experiments.

a) Saturation studies

Fig 1 shows a representative curve of [^{3}H]TBOB binding to rat brain membranes Specific binding was saturable and of high affinity A Scatchard represen-

tation of the saturation isotherm (inset) points to a homogeneous population of binding sites Binding parameters of 16 independent experiments yield a mean equilibrium dissociation constant (K_d) of 7 7 ± 2 0 nM and a total receptor density (R_T) of 22 ± 5 pmol/g tissue corresponding to 0 56 ± 0 12 pmol/mg protein (mean ± SD)

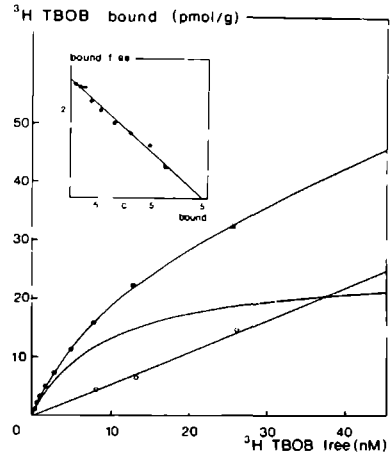

Fig 1
Binding curve of [^{3}H]TBOB to rat brain membranes, showing total binding (●), nonspecific binding (binding in the presence of 10 µM picrotoxin)(○), and specific binding (total binding minus nonspecific binding)(unmarked) as a function of [^{3}H]TBOB concentration Incubations were carried out at 25 °C Incubation time was 45 min, tissue concentration was 25 mg/ml (Each data point is the mean of duplicates, the data are from a representative experiment) Inset Scatchard plot of the binding data

b) Kinetic studies

The association assay was performed at 6 4 nM (n=2) and 3 4 nM (n=2) free [^{3}H]TBOB concentration The dissociation assay was performed once at each of these concentrations Dissociation was initiated by 10 µM picrotoxin or 10 µM TBPS Dissociation curves of specifically bound [^{3}H]TBOB (fig 3) suggest a biphasic dissociation The small fast component (30 % of specific binding) has a dissociation rate constant (k_{-1}) of 1 9x10^{-3} s^{-1} and a half life of dissociation $t_{½}$(diss) of 6 0 min The slower component with a half life of 15 6 min and a dissociation rate constant (k_{-1}) of 7 4x10^{-4} s^{-1} constitutes 70 % of the binding Data points from the two dissociation experiments, each in duplicate, were analyzed simultaneously

The association of [^{3}H]TBOB to rat brain membranes is monophasic (fig 2) The association has a rate constant (k_{+1}) of 1 9x10^5 M^{-1}s^{-1} at 3 4 nM and 2 2x10^6 M^{-1}s^{-1} at 6 4 nM, respectively (the slow component of dissociation was used to estimate k_{+1} according to equation 1) The apparent association half life $t_{½}$(ass)

was 8.4 min at 3 4 nM and 5.3 min at 6.4 nM [^{3}H]TBOB After 28 min and 18 min respectively, 90 % of the maximal receptor occupation was observed. A K_d value of 3.5 nM was calculated from the association and dissociation (slow component) rate constants.

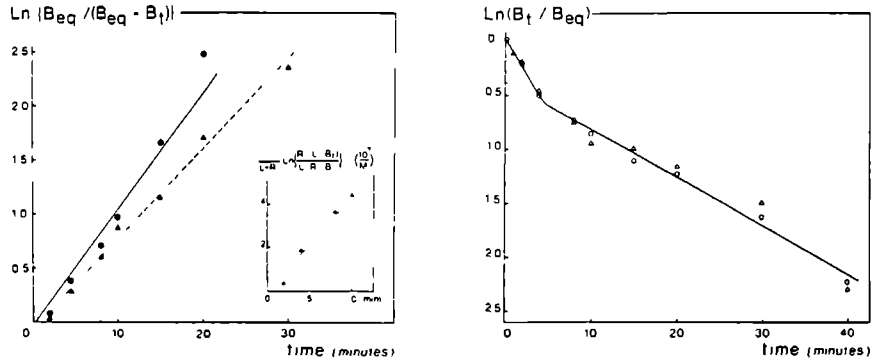

Fig 2
Semi-logarithmic transformation of association curves of [^{3}H]TBOB binding at 6.4 nM, k_{+1}= 2.2x10^6 M^{-1}s^{-1} (●) and 3.4 nM, k_{+1}= 1.9x10^6 M^{-1}s^{-1} (▲) free [^{3}H]TBOB concentration (Each data point is the mean of two experiments in duplicate Monophasic association is seen. Inset: In this transformation the association rate constant is independent of dissociation and is equal to the slope of this line: k_{+1}= 9x10^4 M^{-1}s^{-1} (data are from the experiments for 3 4 nM).

Fig 3
Semi-logarithmic transformation of dissociation curves of [^{3}H]TBOB binding. Identical results are obtained when dissociation is initiated by 10 µM picrotoxin (○) or by 10 µM TBPS (△). Biphasic dissociation is seen Fast component. 30 % of the binding, $t_½$= 6 min, k_{-1}= 1.9x10^{-3} s^{-1}. Slow component: 70 % of the binding, $t_½$= 15 6 min, k_{-1}= 7.4x10^{-4} s^{-1} (Each data point is the mean of duplicates, all data points are analyzed simultaneously)

Inhibition Experiments.

Fig. 4 shows the inhibition of [^{3}H]TBOB binding by GABA, diazepam and TBPS. IC_{50} values of all ligands tested are listed in table 1.

Analysis of [^{3}H]TBOB binding in the absence and presence of the GABAergic facilitating compounds reveals a decrease in the apparent number of binding sites, without a change of the apparent affinity (table 2, fig 5). A decrease in the apparent affinity of [^{3}H]TBOB and a decrease of the R_T were found in the presence of picrotoxin (table 1, fig 5).

Table 1 IC_{50} values of inhibitors of [^{3}H]TBOB binding ([^{3}H]TBOB concentration 4 nM) (mean ± SE)

Compound	n	IC_{50} (µM)	Slope Factor
Muscimol	(5)	2.8 ± 0.8	1.02 ± 0.16
GABA	(4)	8.8 ± 1.5	1.07 ± 0.09
Diazepam	(4)	24 ± 3	0.66 ± 0.04
TBPS	(4)	0.13 ± 0.02	1.12 ± 0.04
Picrotoxin	(11)	0.50 ± 0.03	1.04 ± 0.05
Bicuculline	(3)	enhancement	
βCCE	(2)	enhancement	

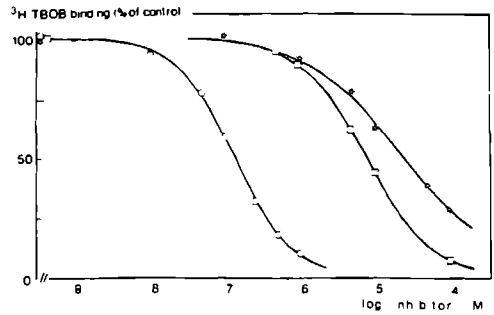

Fig 4
Inhibition curves of specific [^{3}H]TBOB binding by TBPS (○), GABA (□), and diazepam (☆) TBPS and GABA displace [^{3}H]TBOB completely At the highest testable concentration of diazepam, i e 0 1 mM due to limited solubility of the drug, 25 % of the specific binding was still present (Incubation time was 30 min Data points are the mean of duplicates, non specific binding was defined as [^{3}H]TBOB binding in the presence of 10 µM picrotoxin) IC_{50} values are listed in table 1

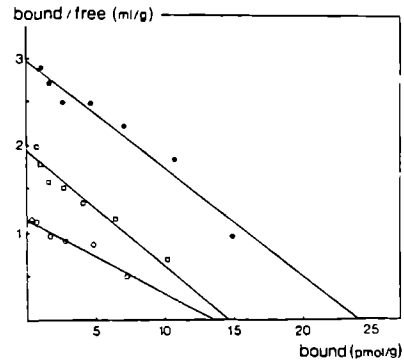

Fig 5
Scatchard analysis of [^{3}H]TBOB binding in the absence of modulating agents (● K_d= 8 4 nM R_T= 24 pmoles/g), in the presence of 5 µM GABA (□ K_d= 7 6 nM, R_T= 15 pmoles/g) and in the presence of 0 5 µM picrotoxin (○ K_d= 11 nM, R_T= 13 pmoles/g) (representative curves of at least four similar results, see table 2, each data point is the mean of duplicates, incubation time is 45 min)

Table 2 Apparent [³H]TBOB binding parameters in the presence of the indicated amounts of inhibiting compounds The saturation experiments were conducted in paired assays with controls in the absence of the inhibiting compound The control values are given in parenthesis Seven concentrations [³H]TBOB varying from 0 1 to 30 nM were used

Compound	concentration	n	K_d (nM) (exp)	K_d (nM) (control)	R_T (pmol/g) (exp)	R_T (pmol/g) (control)
Muscimol	1.10^{-6} M	1	6.6	(7.1)	5	(24)
GABA	5.10^{-6} M	4	8.7	(8.8)	12	(25)
Diazepam	5.10^{-5} M	1	6.4	(7.1)	8	(24)
Picrotoxin	5.10^{-7} M	4	10.2	(7.2)	10	(22)

for n>2 paired Student-t-test
1) GABA n=4 K_d not significantly different from control p>0 1
R_T significantly different from control p<0 01
2) Picrotoxin n=4 K_d significantly different from control p<0 05
R_T significantly different from control p<0 01

DISCUSSION.

Binding Parameters.

The affinity of [³H]TBOB for rat brain membranes was determined in two independent ways by saturation analysis and by analysis of the data from kinetic studies Both independent experimental approaches reveal affinity values of the same magnitude K_d=7 7 nM and K_d(slow diss)=3 5 nM The method to estimate the association rate constant takes into consideration the contribution of dissociation. as is seen in equation 1 If the fast component of dissociation is substituted in equation 1, negative association rate constants are obtained and no corresponding K_d values can be calculated In an alternative analysis of the kinetic data, only datapoints up to the first half life time are taken into account For these points dissociation is still negligible and the following equation holds true

$$\frac{1}{L - R_T} \ln \frac{R_T (L - B_t)}{L (R_T - B_t)} = k_{+1} t \qquad (7)$$

R_T is the receptor concentration and was estimated from the saturation experiments By plotting the left part of equation 7 as a function of time (fig 2, inset) k_{+1} can be estimated to be (0 9 ± 0 3)x10^5 $M^{-1}s^{-1}$ (mean ± SD, n=4), correspond-

ing with K_d values of 8 nM and 20 nM for the slow and fast dissociating component respectively

Saturation analysis (Scatchard plots) indicates only one population of binding sites (K_d=7 7 nM) Kinetic analysis suggests the presence of an additional lower affinity receptor site K_d(fast diss)=20 nM A possible explanation for this disagreement could be that the maximal concentrations [^{3}H]TBOB are too low to reveal the higher K_d Moreover, the affinities as calculated from kinetic studies may be too close to be differentiated by Scatchard analysis

Lawrence et al (1985) found from saturation experiments a K_d value of 60 nM, which is considerably higher than the values reported here In addition these authors report $t_{\frac{1}{2}}$(ass) and $t_{\frac{1}{2}}$(diss)(fast & slow) determined from kinetic experiments but unfortunately they did not determine K_d values from these data Calculating the K_d values from the half lifes given by Lawrence et al (1985) by using the equations 2 4,5 given in Materials and Methods, values of 1 4 nM and 4 nM are obtained These values are not in agreement with the K_d determined from Lawrence's saturation experiments but they are closer to the K_d values reported here

Inhibition studies.

The present study supports the assumption that [^{3}H]TBOB labels a site associated with the $GABA_A$-receptor complex

The GABAergic facilitating agents tested displace [^{3}H]TBOB binding The GABAergic inhibitory ligands bicuculline and βCCE do not displace [^{3}H]TBOB in concentrations up to 0 1 mM, in contrast an enhancement of the binding was observed to 166 ± 5 % (n=3, mean ± SD) of control in the presence of 10 µM bicuculline, to 121 ± 7 % in the presence of 0 1 µM βCCE (n=4, mean ± SD) These observations are in agreement with those of Gee et al , (1986) who demonstrated this effect on [^{35}S]TBPS binding for βCCE Moreover, Squires and Saederup (1987) reported a number of $GABA_A$ receptor blockers to be able to enhance [^{35}S]TBPS binding, in the presence of GABA though As our membrane pellets are washed once only, it is likely that some endogenous GABA is still present in the incubation mixture (Van Rijn et al 1988)

The IC_{50} values of the GABAergic facilitating ligands should be interpreted in view of this probability as well The observed IC_{50} value of GABA is 8 8 µM It is

likely that the allosterically induced changes of TBOB sites are modulated by GABAergic ligands via the low affinity sites of GABA (Ticku and Ramanjanevulu 1984)

The enhancement of [^{3}H]TBOB binding by GABA inhibitory compounds is not mimicked by those GABAergic inhibitory ligands that are thought to act on the convulsant site directly, i e picrotoxin and TBPS, they inhibit [^{3}H]TBOB binding

Saturation analysis shows that the GABAergic facilitating ligands do not affect the apparent affinity of [^{3}H]TBOB, but they appear to decrease the number of binding sites of TBOB such as those of TBPS (Wong et al ,1984) The existence of separate but allosterically interacting, binding sites for TBOB, GABA, and benzodiazepines in the $GABA_A$ receptor complex must be assumed

Picrotoxin lowers the apparent affinity of [^{3}H]TBOB as well as the apparent number of binding sites Two explanations may be possible Picrotoxin binds more rapidly than [^{3}H]TBOB and [^{3}H]TBOB can not completely replace it or picrotoxin and TBOB do not bind to identical sites in a simple competitive manner Tehrani et al (1985) found two binding sites for [^{35}S]TBPS with K_d values of 1 15 nM and 232 nM The IC_{50} value of picrotoxin for the inhibition of [^{3}H]TBOB binding reported here (0 50 µM) corresponds to the IC_{50} value of picrotoxin for the inhibition of low affinity [^{35}S]TBPS sites reported by Tehrani et al (1985) (i e 0 56 µM), suggesting that the picrotoxin binding sites correspond to the described low affinity [^{35}S]TBPS binding sites

The high affinity of [^{3}H]TBOB and its interaction with the above mentioned binding sites makes the ligand very valuable in studying the $GABA_A$ receptor-Cl^--ionophore

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FOLATES: EPILEPTOGENIC EFFECTS AND ENHANCING EFFECTS ON ^{3}H-TBOB BINDING TO THE GABA$_A$ RECEPTOR COMPLEX

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SUMMARY.
The biochemical mechanism responsible for the convulsive effects of folates was investigated The epileptogenic effects of folates were determined in vivo by quantification of the seizures following intracortical application in rats The rank order of epileptogenic effects is
folic acid ≥ 5-HCO-H$_4$folate > H$_2$folate > 5-CH$_3$-H$_4$folate,
This sequence of epileptogenicity in vivo is compared to the rank order of the effects of the above folates on radioligand binding to the GABA$_A$-receptor-complex in vitro The inhibitory potencies of folates on ^{3}H-muscimol and ^{3}H-diazepam bindings did not correlate with their epileptogenic effects However, folates reverse the inhibiting effect of GABA on the binding of the cage convulsant ^{3}H-TBOB (^{3}H-t-butylbicycloorthobenzoate) The rank order of this in vitro effect (folic acid > 5 HCO-H$_4$folate > H$_2$folate = 5-CH$_3$-H$_4$folate), correlates with the rank order of epileptogenicity determined in vivo A relationship between the in vivo and in vitro effects is therefore suggested

INTRODUCTION.

Folic acid can evoke epileptic phenomena when it penetrates into the brain[16, 17, 27, 28] The biochemical background of this action is unknown A direct action of folic acid on synaptic receptors was proposed by Davies and Watkins[8] Hill and Miller[15] provided an electrophysiological indication of such an action, namely the antagonizing of synaptic inhibition Otis et al specified this suggested folate action to a blockade of the GABA response (in electrophysiological studies as well)[31]

Following the suggestions of a direct receptor action of folic acid the epileptic phenomena caused by folic acid were compared to those of known disinhibitory and excitatory substances (Chapter 2) It has been found that the epileptic phenomena induced by folic acid resemble closely those induced by bicuculline and strychnine penicillin[1] and picrotoxin[3] but differ in many respects from those induced by kainic acid[2, 26, 39, 40] carbachol and neostigmine (Chapter 2) These findings support the suggestion that folic acid is blocking the inhibitory system rather than potentiating the excitatory system An additional indication of this hypothesis was reported by Herron et all[13] who found on hippocampal slices a difference of epileptiform responses induced by folic acid and bicuculline compared to kainic acid An effect of folic acid on an excitatory amino acid receptor was suggested by Stephens et all[37] but from this in vivo study a discrimination between excitation and disinhibition on the neuronal level may not be made In conclusion there are indications that folic acid blocks the inhibitory system

GABA (γ-Aminobutyric acid) is considered to be a major inhibitory neurotransmitter in the central nervous system (overview Enna and Karbon[10]) The $GABA_A$-receptor complex is an oligomeric membrane protein with allosteric binding sites[38] Ligand binding assays have demonstrated at least three distinct receptor sites at the receptor complex i e GABA receptor sites (high- and low-affinity), benzodiazepine sites and picrotoxinin or convulsant sites[5, 29] a fourth, a barbiturate recognition site, has been postulated[20, 41]

We tested the influence of folic acid on three components of the $GABA_A$-receptor complex in vitro the high affinity $GABA_A$ site, labeled with ^{3}H-muscimol, the benzodiazepine site, labeled with ^{3}H-diazepam and the picrotoxin- or convulsant site labeled with ^{3}H-TBOB (^{3}H t butylbicycloorthobenzoate)

The epileptogenic effects of folic acid can be evoked by derivatives of folic acid as well[28] Presently we report a sequence of epileptogenicity of four tested folates[4] folic acid 5-formyl-tetrahydrofolate (5-HCO-H_4folate), dihydrofolate (H_2folate) and 5-methyl-tetrahydrofolate (5-CH_3-H_4folate) We compared the rank order of in vivo effects and in vitro effects on the $GABA_A$-receptor complex

MATERIALS AND METHODS.

In vivo experiments.

Subjects.

Male Wistar albino rats were used with a weight of 200 ± 20 g at time of surgery. The animals were individually housed and allowed access to food and water ad libitum. A 12 h light, 12 h dark cycle was maintained. The experiments took place in the light phase.

Surgery.

The animals were anaesthetized by pentobarbital i.p. A polyethylene cannula (outer diameter 0.8 mm, inner diameter 0.4 mm) was implanted through a drill hole in the scull: 1.4 mm to the right of bregma, where the sensorimotor cortex of the left hindleg is situated The cannula was fixed by acrylic cement. The tip of the cannula was 2.0 mm beneath the upper surface of the scull. Histological examination revealed that the tip was in lamina IV or V of the cortex. The cannula could be connected to a flexible injection system, thus permitting free movement during administration of the drugs. The animals were left to recover from surgery for 5 to 7 days.

Observations.

The folates, dissolved in distilled water, were injected through the cannula in a volume of 0.5 - 2.0 µl, with a rate of 0.5 µl/min. One test per day was conducted. Folic acid was tested on all animals, each folate derivative was tested on a group of 8-10 animals, alternated the next day by folic acid which was our control for the day dependency of the response. In a pilot study we had determined the amounts of folate necessary to produce visible jerks. In the present study we tested: for folic acid and 5-HCO-H_4folate: 5 nmoles and 10 nmoles, for H_2folate: 25 nmoles and 50 nmoles, for $5CH_3$-H_4folate: 50 nmoles and 100 nmoles.
The animals were observed for 1.5 h following injection of folate. The observation included the registration of the total duration of the epileptic events, the number of general seizures, the classification of the extension and the intensity of the myoclonic events (see table 1) and the counting of the jerks in four periods of 5 minutes, with intervals of 10 minutes (10-15, 25-30, 40-45 and 55-60 minutes).

In vitro experiments.

Membrane preparation.

Crude synaptic membrane fractions were prepared from whole brains of albino Wistar rats weighing 200 ± 20 g The brains were quickly removed after sacrifice by cervical dislocation and homogenized in 0 32 M sucrose at 1 10 weight/volume ratio with a Potter homogenizer with a teflon pestle for 5 sec at 600 rpm The sucrose homogenates were centrifuged at 1000 g for 10 min (all centrifugations were carried out at 4 °C) The supernatant fractions were decanted (A)

^{3}H-Muscimol and ^{3}H-Diazepam preparations

The supernatant (A) was recentrifuged at 20 000 g for 20 min , the resulting pellet was resuspended in ice-cold water for 15 min (osmotic shock) and then recentrifuged at 8000 g for 20 min The supernatant and the buffy coat were removed, centrifuged at 48,000 g for 20 min and the final pellet was stored at -20 °C for at least 24 hours (B)

^{3}H-Muscimol preparation[9, 14]

After thawing the pellets (B) were treated with 20 volumes of 0 025 % Triton X-100 in 50 mM Tris HCl buffer, pH 7 1, for 30 min at 37 °C and then centrifuged at 50 000 g for 10 min The resulting pellets were washed three times by resuspending them in 50 mM Tris-HCl, pH 7 1 and centrifugation at 50,000 g for 10 min The final pellet was resuspended in the same buffer at a final tissue concentration of 15 mg/ml

^{3}H-Diazepam preparation[6, 7]

After thawing, the pellets (B) were washed three times in 50 mM Tris-HCl buffer, pH 7 7 by centrifugation at 50 000 g for 10 min The final pellet was resuspended in the same buffer at a final tissue concentration of 25 mg/ml

^{3}H-TBOB preparation[19, 34]

The supernatant (A) was centrifuged at 9000 g for 20 min, after which the pellet was washed once by resuspension in 50 mM sodium potassium-phosphate buffer, containing 500 mM NaCl, followed by centrifugation at 16,000 g for 10 min The final pellet was resuspended in the same buffer at a final tissue concentration of 25 mg/ml

Protein was quantified by the method of Lowry[21]

Binding assays

Aliquots of the synaptic membranes suspensions (0 5-1 0 ml) were added to glass tubes together with the radioligand in the absence or presence of nonlabeled test compound

Experiments were run in series with control samples, blanks containing excess of nonlabeled competitive ligand to determine nonspecific binding, and test samples in duplicates or triplicates Incubations were terminated by filtration of aliquots of the incubation mixture through Whatman GF/B filters on a Millipore 12 sample manifold The filters were washed two times with 5 ml ice cold buffer Radioactivity retained on the filters was counted by liquid scintillation spectrometry Experimental data were analyzed and binding parameters calculated by a computer assisted non-linear least square curve fitting routine The kinetic data were analyzed by linear least square curve fitting methods

^{3}H-Muscimol assays[9, 14]

Incubations with ^{3}H-muscimol (spec act 57 Ci/mmol)were performed at 0 °C for 30 min In saturation binding assays, ^{3}H muscimol concentration ranged from 0 5 to 20 nM The inhibition studies as well as the kinetic studies were performed with a constant radioligand concentration of 1 nM Specific ^{3}H muscimol binding was defined as the difference between binding in the absence and presence of 10 µM GABA or 10 µM muscimol and was approximately 80 % of total binding at 1 nM ^{3}H-muscimol

^{3}H-Diazepam assays[6, 7]

The incubation conditions of ^{3}H-diazepam (spec act 85 Ci/mmol) were at first 20 min at 37 °c, then an additional 15 min at 0 °C This procedure increases specific binding[6] In saturation binding assays, ^{3}H-diazepam concentration ranged from 1 0 to 25 nM The inhibition and kinetic studies were performed with a radioligand concentration of 2 5 nM Specific ^{3}H-diazepam binding was determined in the presence of 10 µM unlabeled diazepam and was approximately 85 % of total binding at 2 5 nM radioligand

^{3}H-TBOB assays[19, 34]

Incubations with ^{3}H TBOB (spec act 46 Ci/mmol) were performed at 25 °C for 45 min for binding assays and 30 min for incubation assays In the saturation binding assays the concentrations of ^{3}H-TBOB ranged from 0 1 nM to 100 nM The inhibition and kinetic studies were performed with a radio-

ligand concentration of 4 nM Specific ^{3}H-TBOB binding was defined as the difference between binding in the absence and presence of 10 µM picrotoxin or 10 µM TBPS and was 60-70 % of total binding at 4 nM ^{3}H-TBOB

Concentration-response curves were made for the folates in the absence and presence of 5 µM GABA Six or seven concentrations of the folates were used, varying from 1 µM to 1 mM

Chemicals.

The radioligands were obtained from Amersham, England The nonlabeled ligands were obtained from Sigma England except for TBPS, which was delivered by NEN Germany

RESULTS 1 : In vivo experiments.

Behavioral effects of the folates in general.

Injection of the described amounts of the various folates into the sensorimotor cortex of the rat produced muscular contractions We observed no abnormalities after injecting a physiological NaCl solution The muscular contractions were clearly observable myoclonic jerks, varying from a slight extension of one of the toes to an abrupt strong flexion movement in knee and hip combined by axial turning The extension of the jerks was sometimes restricted to the hindleg, but often the forelimb, ear and eyelid were involved too Contralateral jerking was seen as well The spread and intensity of the jerks could not be quantified reliably, due to their continuously changing pattern Median values of the maximal intensity- and extension values are given in table 2

Apart from the myoclonic jerks some generalized seizures were seen, lasting a few seconds only (table 2) No other behavioral abnormalities were observed consciousness was unimpaired and grooming behavior was normal (the generalized seizures excepted)

Behavioral effects of the folates: rank order.

The jerking started within 5 minutes after injection, lasting for about one hour (table 2) A significant difference in duration between folic acid and 5-HCO-H_4folate at the 5 nmoles dose but not at the 10 nmoles dose was observed (Wilcoxon test at the $p=0.05$ level)

The number of jerks is given in table 2 The results of folic acid on the first test day are given No difference between the equimolar doses of folic acid and 5-HCO-H_4folate was observed and no difference between data of folic acid on the first day and folic acid on the control days was observed (data not shown) (Wilcoxon $p>0.05$) A rank order of epileptogenic effects was derived from table 2

Folic acid $\geq$ 5-HCO-H_4folate > H_2folate > 5-CH_3-H_4folate

Table 1 Classification criteria of the myoclonic contractions

Extension Class/visible motor effect in:	Intensity Class/motor effect resulting in:
1 only left hindlimb	1 jerking without lifting the limb
2 left hindlimb and forelimb	2 jerking with lifting the limb
3 both left limbs + face	3 associated axial turning
4 contralateral	4 rolling on the back

Table 2 Classification and quantification of the epileptic phenomena

folate	n	doses (nmoles)	max intensity class median (range)	max extension class median (range)
Folic acid	28	5 , 10	2 (1-3) , 3 (1-4)	2 (1-3) , 3 (1-3)
5-HCO-H_4folate	8	5 , 10	2 (2-3) , 3 (2-3)	2 (1-3) , 3 (1-3)
H_2folate	10	25 , 50	2 (1-3) , 2 (1-4)	1 (1-3) , 2 (1-3)
5-CH_3-H_4folate	10	50 ,100	2 (1-3) , 1 (1-3)	1 (1 3) , 1 (1-3)

folate	duration of jerks (minutes) mean±SD	number of jerks (#/20 min) mean±SD	generalized seizures (# rats/# group)
Folic acid	60 ± 14 , 76 ± 18	315 ± 205 , 527 ± 235	2/8 , 4/8
5-HCO-H_4folate	44 ± 16 , 66 ± 17	273 ± 166 , 571 ± 175	1/8 , 4/8
H_2folate	54 ± 17 , 75 ± 22	213 ± 141 , 302 ± 97	0/10 , 2/10
5-CH_3-H_4folate	45 ± 21 , 53 ± 19	98 ± 52 , 202 ± 148	0/10 , 0/10

RESULTS 2 : In vitro experiments.

Binding-parameters of the radioligands.

Saturation analysis reveals nM affinity binding of ^{3}H-muscimol (K_d 5.2 ± 1.3 nM, mean ± SEM, n=10), ^{3}H-diazepam (K_d 17 ± 5 nM, n-7) and ^{3}H-TBOB (K_d 7.7 ± 2 0 nM, n=16). The K_d values found are in general agreement with previously published reports[6, 7, 9, 14, 44], except for ^{3}H-TBOB The K_d value of ^{3}H-TBOB appears to be lower than the result reported before[19] (i.e. 60 nM). In a separate study in which the binding behavior of ^{3}H-TBOB was evaluated in detail, the low K_d value has been confirmed (Chapter 5). Displacement experiments with known GABA-ergic compounds as well as enhancement data on ^{3}H diazepam binding are consistent with previous reports as well[6, 7, 29]. The results of the displacement studies are given in table 3. GABA and muscimol enhanced the binding of 3H-diazepam: GABA (50 µM) to a maximum of 150 ± 7 % of initial binding (mean ± SEM, n=8), muscimol to 190 % of initial binding (n=1). Pentobarbital enhanced the binding of ^{3}H-diazepam as well, to 180 % of initial binding at the highest dissolvable concentration of 1 mM, n=1.

Table 3. IC_{50} and EC_{50} values of tested compounds. (Legends see next page)

Ligand	1 nM ^{3}H-Muscimol			2.5 nM ^{3}H-Diazepam			4 nM ^{3}H-TBOB		
Compound	µM	%SD	n	µM	%SD	n	µM	%SD	n
Muscimol	.0035		1	↑ .5		1	1.1	18	5
GABA	.049	42	6	↑ 1.4	21	5	7.7	19	4
Bicuculline	3.8	18	3	>100		1	↑ .18	17	3
Bicuculline^{M}Cl	34		1	>100		1	↑ .20		1
Diazepam	>100		1	.014	36	7	39	5	4
βCCE	nd			.0013	43	4	↑ .02	50	3
Picrotoxin	>1000		1	>1000		2	.50	6	11
TBPS	nd			nd			.10	22	5
Pentobarbital	nd			↑ *		2	41	12	2
Folic acid	220	18	4	230	17	6	↑ *		20
5-HCO-H_4folate			2	1476		2	↑ *		10
H_2folate			2	230		2	↑ *		5
5-CH_3-H_4folate			2	1467	17	1	↑ *		5

Table 3
Displacement IC_{50} values and enhancement EC_{50} values of the tested compounds for the three radioligands used Binding conditions are described in Materials and Methods
Values are given in µM SD the standard deviation given as a percentage of the IC_{50} value n the number of experiments in triplicates (^{3}H-muscimol) or duplicates (^{3}H-diazepam and ^{3}H-TBOB) nd not determined
*↑ no inhibition but an enhancement of the radioligand binding is observed * no saturation was observed at the highest dissolvable concentration i e 1 mM The data of the folates for ^{3}H-muscimol were analyzed simultaneously (see fig 1) For ^{3}H-diazepam the data of folic acid and H_2folate were analyzed simultaneously as well as the data of 5 HCO-H_4folate and 5-CH_3-H_4folate (see fig 2)*

Folate influence on the binding of the radioligands.

- The folates did displace bound ^{3}H-muscimol but no significant difference between the folates was observed (Analysis of variance $F_{3,64} = 0.38$, $p>0.7$, fig 1, table 3) The IC_{50} was 220 µM the slope factor was 0 64

The folates did displace ^{3}H-diazepam binding as well A significant difference between the folates was found ($F_{3,68} = 8.70$ $p<0.05$) Scheffe's post hoc analysis revealed no difference between folic acid and H_2folate (IC_{50} 230 µM, slope factor 0 62) and no difference between 5-HCO-H_4folate and 5-CH_3-H_4folate (IC_{50} 1476 µM, slope factor 0 68) (fig 2, table 3)

- GABAergic facilitating ligands displaced bound 3H-TBOB with micromolar potencies The convulsants TBPS and picrotoxin displaced the radioligand as well The GABAergic inhibitory compounds bicuculline (a $GABA_A$ antagonist) and βCCE (an inverse agonist of benzodiazepine receptors) however enhanced the binding of ^{3}H TBOB Bicuculline (5 µM) enhanced the binding to 169 ± 4 % of initial binding (mean ± SEM, n=3) and βCCE (1 µM) to 118 ± 5 % (n=3)(table 3) Higher concentrations of bicuculline and βCCE inhibited ^{3}H-TBOB binding (data see Ch 8)

The folates enhanced the binding of ^{3}H-TBOB as well (fig 3) In the absence of GABA this enhancement was obvious for folic acid at a concentration of 0,5 mM folic acid the binding is 140 ± 6 %, n=28, mean ± SEM) For the other folates tested the enhancement was less A significant difference between the folates was found ($F_{3,184} = 12.12$ $p<0.01$) In Scheffe's post hoc analysis folic acid differed from the other three folates at the $p=0.05$ level No difference between the latter three folates was found

The enhancing effects of the folates increased relatively in the presence of GABA In the presence of 5 µM GABA (initial binding was 65 % of control, fig 4

left ordinate) folic acid produced an enhancement to 220 % of initial binding (fig 4, right ordinate) The enhancing effect of 5-HCO-H_4folate under this condition was slightly less 190 % whereas the enhancements by H_2folate and 5-CH_3-H_4folate were less, reaching a 140 % of GABA control only Analysis of variance has been performed The difference between the folates is highly significant ($F_{3\,75} = 73\,46$, $p<0\,001$) Post hoc analysis reveals a difference between folic acid and the other three folates as well as a difference between 5-HCO-H_4folate and the other folates No difference between H_2folate and 5-CH_3-H_4folate was observed

In conclusion the rank order of ^{3}H-TBOB enhancing effect of the folates in the presence of GABA

Folic acid > 5-HCO-H_4folate > H_2folate = 5-CH_3 H_4folate

Fig 1
Displacement curves of specific ^{3}H-muscimol binding by folic acid (●), 5-HCO-H_4folate (○), H_2folate (■) and 5-CH_3-H_4folate (□) Each data point is the mean of at least two independent measurements in triplicate see table 3, bar is 1 x SD No significant difference between the folates was observed analysis of variance $F_{3,64} = 0\,38$, $p>0\,7$ IC50 220 µM, Slope factor 0 64

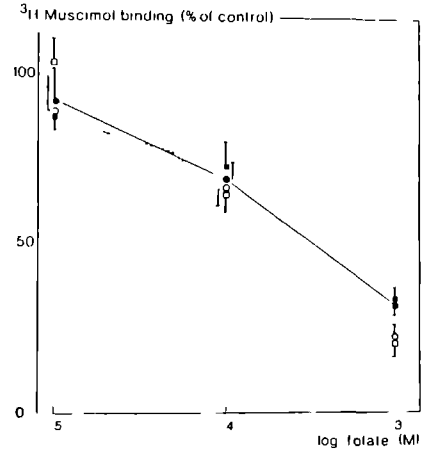


Fig 2
Displacement curves of specific ^{3}H-diazepam binding by folic acid (●), 5-HCO-H_4folate (○), H_2folate (■) and 5-CH_3-H_4folate (□) Each data point is the mean of at least two independent measurements in duplicate see table 3, bar is 1 x SD No significant difference between folic acid and H_2folate was observed IC_{50} 230 µM, Slope factor 0 62 A significant difference between folic acid compared with 5-HCO-H_4folate and 5-CH_3-H_4folate was observed IC_{50} of the latter two 1467 µM, Slope factor 0 68 Analysis of variance $F_{3,68} = 8\,70$, $p<0\,05$) and Scheffe's post hoc analysis IC_{50} values are listed in table 3

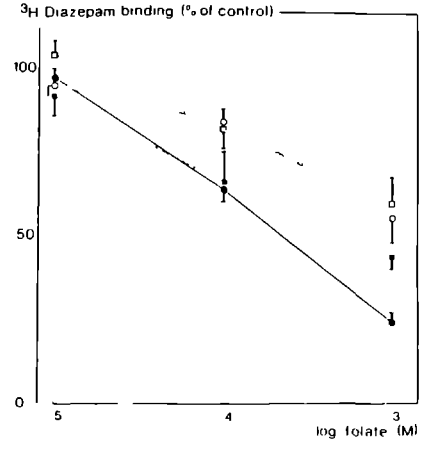


Fig. 3
Effect of various concentrations of folic acid (●), 5-HCO-H_4folate (○),H_2folate (■) and 5-CH_3-H_4folate (□) on ^{3}H-TBOB binding (4 nM) in the absence of exogenous GABA. Each data point is the mean of at least five independent measurements in duplicate, bar is 1 x SD. The folates enhance ^{3}H-TBOB binding ($F_{5,184}$ = 6.11; $p<0.01$).
A significant difference between the folates was found ($F_{3,184}$ = 12.12; $p<0.01$). In Scheffe's post hoc analysis folic acid differed from the other three folates at the $p=0.05$ level. No difference between the latter three folates was found.

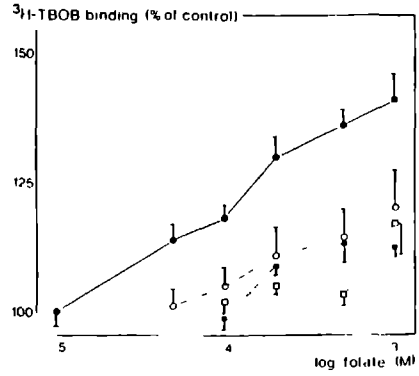
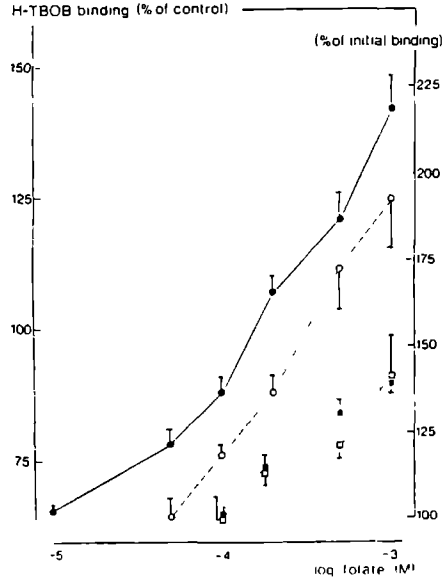


Fig. 4
Effect of various concentrations of the folates on 3H-TBOB binding (4 nM) in the presence of 5 µM GABA (65 % of control binding)(folic acid (●), 5-HCO-H_4folate (○),H_2folate (■) and 5-CH_3-H_4folate (□)). Each data point is the mean of four independent measurements in duplicates, bar is 1 x SD.
The folates differed significantly ($F_{3,75}$ = 73.46; $p<0.001$). Post hoc analysis revealed a difference between folic acid, 5-HCO-H_4folate and the other two folates. No difference between H_2folate and 5-CH_3-H_4folate was seen.



DISCUSSION

In the rat intracortical application of folic acid produces muscular contractions We have shown by ECoG recordings that these motor phenomena resemble epileptic events[1] (Ch 2) The epileptic events can be evoked by folate derivatives as well The rank order of folates to induce the epileptogenic jerks we report is in general agreement with our previous studies, in which we used a different technique to induce and to quantify the epileptic phenomena[28]

The present study tried to determine whether the $GABA_A$ receptor complex is the primary site of interaction responsible for the epileptogenic action of the folates

Folates displace both ^{3}H-muscimol and ^{3}H-diazepam with comparable affinities and no clear differentiation between the folates is found in interaction with either of these binding sites It is therefore not likely that an interaction of the folates with the high affinity $GABA_A$ site or with the benzodiazepine site of the $GABA_A$ receptor complex can account for the epileptic phenomena

In chapter 7 we will show that folic acid reverses the inhibitory effect of GABA on ^{3}H-TBOB binding[42] Here we report that all four tested folates reverse the GABA suppression of ^{3}H-TBOB binding Moreover, the rank order of epileptogenic activity in vivo corresponds to the rank order in reversing the inhibitory action of GABA in vitro on ^{3}H-TBOB binding Therefore it seems reasonable to assume that the enhancement of ^{3}H-TBOB binding by folates in the presence of µmolar GABA can account for the epileptic phenomena

This assumption is supported by the following observations

1 The folate concentrations reversing the inhibitory effect of GABA on ^{3}H-TBOB binding (10 µM to 1000 µM) are in agreement with the effective folate concentration measured in vivo following intracortical injection of 5 nmoles of folic acid the concentration in the brain near the cannula is 20 µM -100 µM (Ch 2), the folic acid concentration after intravenous injection (after a focal lesion of the blood brain barrier) has a value of 150 µM in the focus[17]

2 Herron et al needed a bath concentration of 500 µM folate to evoke population spikes in hippocampal slices[13]

From 1 and 2 we conclude that the epileptogenic folate concentration in vivo is in agreement with the effective concentration in vitro

3 The concentration of GABA that has to be added to observe the rank order of in vitro effects of the folates is of the same range as the concentration of GABA in functional experiments GABA increases the Cl^- permeability at a con centration of about 10 µM[18]

4 Folate induced seizures resemble those induced by bicuculline, a ligand labeling selectively the low affinity $GABA_A$ sites having micromolar affinity for GABA (K_d ~ 1µM)[30]

5 The presence of anions such as Cl^- remove the high affinity population of GABA binding sites, leaving the low affinity population[22] We used 500 mM NaCl to optimize ^{3}H-TBOB binding[34, 36]

From 3 - 5 we conclude that the $GABA_A$ low affinity binding site is probably influenced by the folates

^{3}H-TBOB was only recently introduced, only a few reports on interactions with the other binding sites of the $GABA_A$ complex are available yet[19, 25, 33] The effects may be compared to reports on ^{35}S-TBPS binding interactions[23, 24, 32, 43] A number of GABAergic inhibitory compounds in electrophysiological systems reverse (partially) GABA suppression of ^{35}S-TBPS binding[11, 12, 35] It has been suggested that reversal of the inhibitory effect of GABA on ^{35}S TBPS binding is a method for assessing the seizure inducing liability of drugs[35, 36] The results of the present study support this suggestion

CONCLUSION.

The rank order correlation of the tested folates for the ability to enhance ^{3}H-TBOB binding to rat brain membranes in vitro in the presence of GABA and to induce myoclonic jerks after intracortical application, suggest a possible relationship between folate induced receptor interactions within the $GABA_A$ receptor complex and folate induced epileptic phenomena

Acknowledgements

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THE INFLUENCE OF FOLIC ACID ON THE PICROTOXIN SENSITIVE (CONVULSANT) SITE OF THE GABA$_A$ RECEPTOR COMPLEX

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SUMMARY.

The site of action responsible for the convulsive effect of folic acid was investigated in vitro Folic acid (EC_{max} $5x10^{-4}$ M) enhances the binding of the cage convulsant [^{3}H]-t-butylbicycloorthobenzoate (^{3}H-TBOB) to rat brain membranes, namely to 130 % of control in the absence of GABA and to over 300 % of control in the presence of physiological concentrations of GABA Analysis of the binding parameters reveals that folic acid increases the apparent number of ^{3}H TBOB binding sites

INTRODUCTION

Folic acid can evoke epileptic phenomena when it penetrates into the brain, as is known since the sixties [3,7] The biochemical background of this action is still unknown A direct action of folic acid on synaptic receptors was proposed by Davies and Watkins in 1973 [1] The very next year, Hill and Miller [2] provided an electrophysiological indication of such an action, namely the antagonizing of synaptic inhibition It was not until 1985 that a second report with electrophysiological evidence of a direct synaptic action of folic acid was published a blockade of the GABA response [8] The finding that the epileptic phenomena induced by folic acid resemble closely those induced by the GABA-antagonist bicuculline [Ch 2], but differ in many respects from those induced by the excitatory kainic acid [11], supports the suggestion that folic acid affects the inhibitory GABA system Recently an effect of folic acid of a different nature, viz on an excitatory amino acid receptor was reported [10], but this in vivo study cannot exclude disinhibition on the neuronal level We tested the influence of folic acid on various

components of the GABA receptor-Cl-ionophore (Ch 6) We report here the influence of folic acid on the convulsant site of the complex

METHODS.

The characterization of the convulsant (picrotoxin sensitive) site was performed according to Lawrence et al [6] with [^{3}H]-t-butylbicycloorthobenzoate (^{3}H-TBOB, specific activity 54 Ci/mmole, Amersham) Homogenates of brains of male Wistar rats (200 ± 20 g) were used Crude synaptic membranes (P2 pellets) were suspended in 0 05 M sodium-potassium-phosphate buffer pH 7 4, containing 0 5 M NaCl [6,9] In this buffer the pellets were washed once ^{3}H TBOB (± $4x10^{-9}$ M) was incubated in the absence or presence of varying concentrations of folic acid GABA, or both (Sigma) Saturation binding assays with ^{3}H-TBOB concentrations varying from $0\ 1x0^{-9}$ M to $30x10^{-9}$ M, were performed in the absence or presence of constant concentrations of folic acid and/or GABA

Incubations were at 25 °C for 30 min (inhibition studies) or 45 min (saturation studies) Nonspecific binding was defined as binding of ^{3}H-TBOB in the presence of 10^{-5} M picrotoxin

The binding data were analyzed using computer assisted nonlinear regression analysis methods

RESULTS AND DISCUSSION.

As the interaction of folic acid with the picrotoxin sensitive site seems to be of an allosteric nature (see below), we investigated the interaction between the three substances folic acid, GABA and TBOB by means of ^{3}H-TBOB receptor binding studies in three different, partly complementary, settings keeping the concentration of two compounds constant while varying the concentration of the third

In the first setting the folic acid concentration was varied at a constant ^{3}H-TBOB concentration of $4x10^{-9}$ M and in the absence or presence of $5x10^{-6}$ M

or 5x10^{-5} M GABA (fig 1) Variation of folic acid concentration produces a dose-dependent enhancement of ^{3}H-TBOB binding In the absence of GABA (control), 5x10^{-4} M folic acid produces an enhancement to (132 ± 4) % (mean ± SEM, n=7 fig 1, left ordinate) The enhancing potency of folic acid increases relatively in the presence of GABA In the presence of 5x10^{-6} M GABA, folic acid produces an enhancement to 190 % of initial binding (fig 1, 2nd ordinate, initial binding 70 % of control) In the presence of 5x10^{-5} M GABA the enhancement is as high as 380 % (at 10^{-3} M folic acid, fig 1, 3rd ordinate, initial binding is 20 % of control) The effects of concentrations of folic acid in excess of 10^{-3} M could not be determined due to limited solubility of the compound

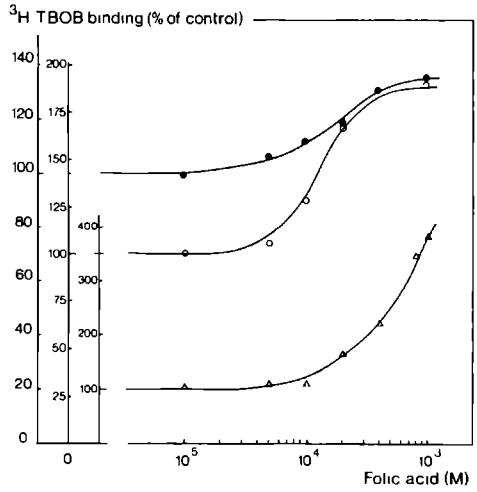

Fig 1
Effect of varying the concentration of folic acid on ^{3}H-TBOB binding (4x10^{-9} M) in the absence (● left ordinate, is control) and in the presence of 5x10^{-6} M GABA (○ ,middle ordinate initial binding is 70 % of control) and 5x10^{-5} M GABA (△ ,right ordinate initial binding is 20 % of control) (each data point is the mean of duplicates, representative curves of three similar experiments) Note folic acid reverses the inhibitory effect of GABA on the binding of ^{3}H-TBOB

Hommes [4] demonstrated that the concentration of folic acid in the epileptogenic focus after intravenous injection of folic acid (in rats) at the moment spikes become visible on the EEG, has a value of 1 5x10^{-4} M The concentration range of folic acid that results in an enhancement of ^{3}H-TBOB binding is thus in agreement with the result of these in vivo experiments

Physiological concentrations of GABA are in the micromolar range Moreover GABA increases the CL^- permeability at a concentration of around 10^{-5} M [5] It is therefore reasonable to assume that the enhancement of ^{3}H-TBOB binding by folic acid in the presence of ± 10^{-5} M GABA can account for a pharmacologically

relevant response Effects of the same kind on ^{35}S-TBPS binding were recently reported for some GABA antagonists [9]

In the second setting the GABA concentration was varied at a constant ^{3}H-TBOB concentration of $4x10^{-9}$ M and in the absence or presence of $5x10^{-4}$ M folic acid (fig 2) GABA inhibits ^{3}H-TBOB binding with an IC_{50} of $(5\,5 \pm 0\,3)x10^{-6}$ M (mean ± SD, n=2) The enhancement of ^{3}H-TBOB binding produced by folic acid can be reversed, since GABA inhibits the enhanced binding as well, but the potency of GABA in inhibiting the ^{3}H-TBOB binding is reduced by folic acid the IC_{50} of GABA increases threefold, $IC_{50} = (18 \pm 8)x10^{-6}$ M (n=2)

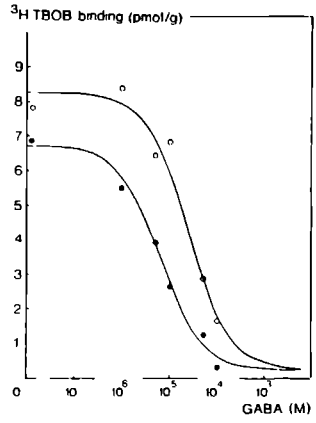

Fig 2
Effect of varying the concentration of GABA on ^{3}H-TBOB binding (4 nM) in the absence (●) and in the presence (○) of $5x10^{-4}$ M folic acid (data points are the mean of duplicates, this result was replicated once) Note folic acid enhances the IC_{50} of GABA threefold

In the third setting the ^{3}H-TBOB concentration was varied in the absence or presence of $5x10^{-4}$ M folic acid, or $5x10^{-6}$ M GABA, or both (fig 3, binding parameters table 1) Folic acid produces an increase in the apparent number of binding sites In contrast the apparent affinity is unchanged The effect of GABA is found to be a reduction of the apparent number of binding sites, again without an effect on the affinity Incubation of the samples in the presence of both folic acid and GABA produces a parallel Scatchard plot as well Once again it is seen that the effects of folic acid and GABA are opposite to each other, which is shown in the first two settings as well

Table 1 Binding parameters of ^{3}H-TBOB in the absence and in the presence of folic acid or GABA, (mean ± SEM)
K_d values are not significantly different by paired Student t-test ($p > 0.05$)
R_t values do significantly differ from each other by paired Student t-test ($p < 0.01$)

compound		n	K_d nM	R_t pmol/g
control		9	7.4 ± 0.8	21 ± 2
Folic acid	$5x10^{-4}$ M	9	8.7 ± 0.9	34 ± 3
GABA	$5x10^{-6}$ M	4	8.7 ± 1.3	12 ± 2

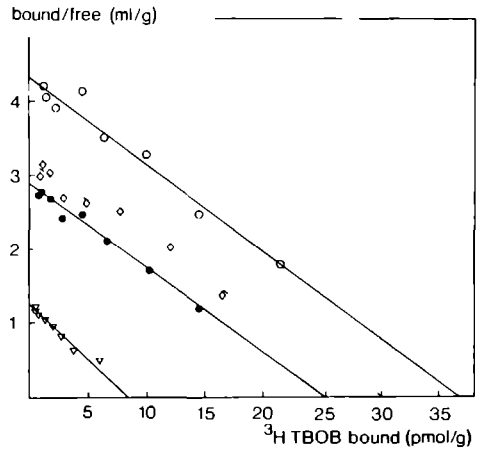

Fig 3
Scatchard analysis of ^{3}H-TBOB binding in the absence of modulating agents (●), in the presence of $5x10^{-4}$ M folic acid (○) in the presence of $5x10^{-6}$ M GABA (▽)(representative curves of at least four similar results, see table) and in the presence of both $5x10^{-4}$ M folic acid and $5x10^{-6}$ M GABA (◇) Note folic acid enhances the apparent R_t whereas GABA reduces it Combined addition of both agents results in an intermediate R_t value

The enhancement of ^{3}H-TBOB binding by folic acid may be interpreted as opposing GABA's binding-diminishing action In this way the disinhibitory properties of folic acid found in electrophysiological experiments [2,8] can be translated into terms of receptor binding studies Since the membranes are washed once only, we might speculate that the enhancing effect of folic acid in the control situation might be due to the presence of some endogenous GABA

The mechanism of this folic acid effect is clearly not picrotoxin like, i e competitive with ^{3}H-TBOB, but seems to be of an allosteric nature Further experiments are needed to elucidate the point of interaction of folic acid on the $GABA_A$-receptor complex-Cl^-ionophore

CONCLUSION.

The presented results suggest that folic acid does indeed have an influence on the $GABA_A$ receptor complex, namely a modulation of the picrotoxin sensitive site In doing so folic acid reverses the action of GABA The exact mechanism of this effect is still obscure

It is tempting to assume that this biochemical effect might be a first step in the elucidation of the mechanism of folic acid induced epileptic phenomena

Acknowledgements.

Drs J P C Zwart, Prof Dr H Meinardi and Mrs H L M Siero are gratefully acknowledged for their constructive discussions (Grant TNO-CLEO A50)

A COMPARISON OF THE EFFECTS OF FOLIC ACID, BICUCULLINE AND ETHYL-β-CARBOLINE-3-CARBOXYLATE ON ^{3}H-TBOB BINDING

With the participation of
J F Rodrigues de Miranda, T J A M van der Velden,
E Willems-van Bree and O R Hommes

SUMMARY.
We compared folic acid with bicuculline and ethyl-β-carboline-3-carboxylate (βCCE) in influencing the binding of the cage convulsant [^{3}H]-t-butylbicycloorthobenzoate (^{3}H TBOB) to rat brain membranes Folic acid (10^{-5} M to 10^{-3} M) enhances ^{3}H-TBOB binding and reverses the inhibitory effect of GABA and diazepam, but not that of TBPS and picrotoxin, on ^{3}H-TBOB binding Bicuculline (10^{-8} M to 10^{-5} M), but not βCCE, mimics the influence of folic acid on ^{3}H-TBOB binding These findings support the hypothesis that seizures induced by folic acid and by bicuculline share a common biochemical mechanism

INTRODUCTION

Folic acid can evoke epileptic phenomena when it penetrates into the brain [20] The biochemical background of this action is unknown [6] A blockade of the inhibitory GABA response was found in electrophysiological experiments [22], suggesting an effect of folic acid on the $GABA_A$ receptor complex

The $GABA_A$ receptor-complex is an oligomeric membrane protein with allosteric binding sites [1] Ligand binding assays have demonstrated at least three distinct binding sites on the receptor complex GABA receptor sites (high- and low-affinity), benzodiazepine sites and picrotoxinin or convulsive sites [1] ^{3}H-TBOB is thought to label a site closely related to the picrotoxinin binding site [27]

In the Chapters 6 and 7 we reported that folic acid reverses the action of GABA on ^{3}H-TBOB binding [31] Presently we report that this action of folic acid resembles closely that of bicuculline a $GABA_A$ antagonist [21], but not that of ethyl-β-carboline 3-carboxylate (βCCE) an inverse-agonist of benzodiazepine binding sites [3]

METHODS.

[^{3}H]-t-butylbicycloorthobenzoate binding was performed according to Lawrence et al [9] See also chapter 5 (^{3}H-TBOB, specific activity 54 Ci/mmole) Homogenates of brains of male Wistar rats (200 ± 20 g) were used Crude synaptic membranes (P2 fractions) were suspended in 0 05 M sodium-potassium-phosphate buffer, pH 7 4, containing 0 5 M NaCl The pellets were washed once only ^{3}H-TBOB (4 nM) was incubated in the absence or presence of fixed concentrations of GABA, diazepam, or TBPS, and varying concentrations of folic acid bicuculline or βCCE Incubations were at 25 °C for 45 min Nonspecific binding was defined as binding of ^{3}H-TBOB in the presence of 10 µM picrotoxin The binding data are given as the fraction of control binding, i e the binding in the absence of any modulating drugs

The radioligand was obtained from Amersham, England The nonlabeled ligands were obtained from Sigma, England, except for TBPS, which was delivered by NEN, Germany

RESULTS.

GABA (IC_{50} 8 8 ± 1 5 µM, slope factor 1 07 ± 0 09), diazepam (IC_{50} 24 ± 3 µM, slope factor 0 66 ± 0 04) and TBPS (IC_{50} 0 13 ± 0 02 µM, slope factor 1 12 ± 0 04)(mean ± SEM, n=4), displaced ^{3}H-TBOB binding to rat brain membranes

Folic acid and bicuculline both produced a dose dependent enhancement of 4 nM ^{3}H-TBOB binding, as is depicted in fig 1 (folic acid) and in fig 2 (bicuculline) In the absence of other drugs folic acid produced an enhancement to 143 ± 5 % of control binding (mean ± SD, n=20) at the highest concentration tested (1 mM),

whereas the enhancement by 10 µM bicuculline reached 171 ± 10 % of control binding (n-8). βCCE altered ^{3}H-TBOB binding in a biphasic manner, reaching 125 ± 5 % of control binding at 0.1 µM and 76 ± 6 % at 10 µM (n = 4).

Both folic acid and bicuculline reversed the inhibition of ^{3}H-TBOB binding by GABA as well as the inhibition by diazepam. The inhibition caused by 5 µM GABA was completely reversed, whereas the influence of 50 µM GABA (tested only with folic acid, data not shown) or with 50 µM diazepam was reversed only partially. βCCE reversed neither the inhibition of GABA nor that of diazepam.

The inhibition of ^{3}H-TBOB by TBPS (a compound structurally related to TBOB) was not reversed by either of the tested ligands.

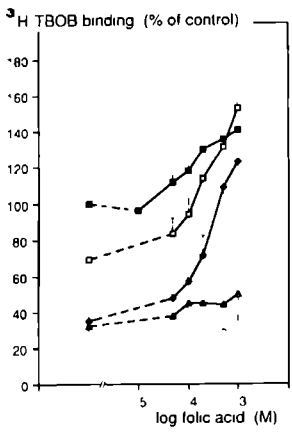

Fig. 1

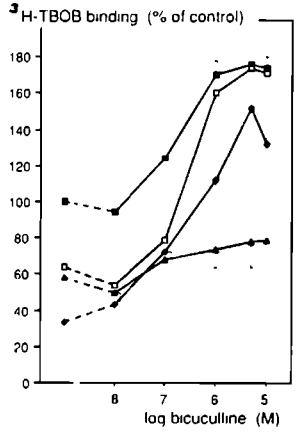

Fig. 2

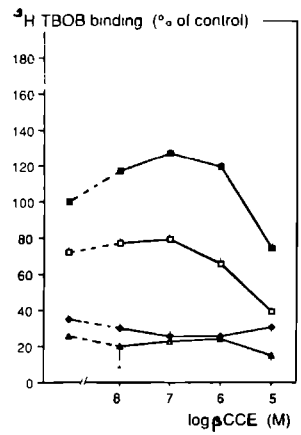

Fig. 3

Fig. 1, 2, 3.
Effect of varying the concentration of folic acid (fig.1), bicuculline (fig.2) and βCCE (fig.3) on ^{3}H-TBOB binding (4 nM) in the absence of any modulating agents (■), in the presence of 5 µM GABA (□), in the presence of 50 µM diazepam (◆) and in the presence of 0.2 µM TBPS (△).
Each data point is the mean of at least 4 measurements. The bars indicate 1xSD.
Note: the pattern of influence on the binding of the radioligand of folic acid resembles that of bicuculline.

DISCUSSION.

^{3}H TBOB was only recently introduced as a probe for the $GABA_A$ receptor coupled Cl^--channel Only a few reports for its interactions with the other binding sites of the $GABA_A$-receptor complex are available yet [2, 9, 15, 26] As numerous reports on allosteric interactions of the ^{35}S TBPS binding sites with the other sites of the $GABA_A$ complex are published [e g 4, 5, 7, 8, 32, 12, 13, 17, 19, 24, 27, 28, 29 30] we compare our results to these although it is suggested that the compounds TBOB and TBPS may have slightly different influences on the ion channel [19, 23] The binding site of picrotoxin is thought to be closely related to the recognition site(s) of TBOB and TBPS [27]

It is suggested that GABA and GABAergic enhancing ligands modulate the convulsant binding sites via an allosteric action In our membrane preparations, GABA and diazepam inhibit ^{3}H-TBOB binding Some previous reports show an enhancement of ^{35}S-TBPS binding by diazepam [32, 17, 30] However, these studies used extensively washed membrane preparations It is assumed that displacement of ^{35}S-TBPS binding by diazepam is GABA dependent and that in GABA free preparations an enhancement of ^{35}S-TBPS binding by diazepam is found [8] As our membranes are washed once only, the presence of endogenous GABA may account for the observed inhibition [8, 12, 13] Moreover, the enhancement of ^{3}H-TBOB binding by bicuculline and by folic acid in the absence of exogenous modulators are likely be due to unwashed endogenous GABA as well [33]

Folic acid and bicuculline reverse allosteric inhibition of ^{3}H-TBOB binding but did not affect (competitive) inhibition by the convulsant TBPS Folic acid appeared to reverse the inhibitory effect of pentobarbital as well, but not that of picrotoxin (data not shown, we have not tested this for bicuculline but it has been reported for bicuculline on ^{35}S-TBPS binding in [10, 11]) These results suggest that folic acid and bicuculline may affect in a similar way the $GABA_A$ receptor-complex This suggestion is supported by in vivo experiments When injected into the neocortex of the rat, folic acid produces epileptic phenomena which are in many respects similar to the syndrome induced by bicuculline methylchloride

A number of convulsant drugs have been shown to reverse GABA inhibition of ^{35}S-TBPS binding [7, 28] Some endogenous amino acids, associated with seiz-

ures, have this effect as well [29] It is suggested that the ability of a drug to reverse GABA's inhibitory effect on TBPS binding may predict convulsive effects [28]

The pattern of reversing the inhibition of ^{3}H-TBOB binding by folic acid and bicuculline is not mimicked by βCCE The biphasic effect of βCCE on control binding is in agreement with the results of reported functional experiments [25] GABA induced Cl^{-}- currents changed from 12 % inhibition at 0 1 µM βCCE to 30 % stimulation at 10 µM βCCE Other authors report an inhibition of ^{35}S-TBPS binding [16, 18] or an enhancement of this binding [5] The lack of interaction between βCCE and diazepam is somewhat surprising, as these ligands are thought to compete for the same receptor sites The concentrations of diazepam and βCCE needed to produce their effects on the binding of ^{3}H-TBOB were above 10^{-7} M These concentrations do not correspond with the nanomolar affinities reported for binding to the high affinity central benzodiazepine receptor site [3, 14] This discrepancy pleads against an interaction of the nanomolar affinity benzodiazepine receptor sites (binding diazepam as well as βCCE) with the ^{3}H-TBOB sites

CONCLUSION.

The aim of this study was to investigate the biochemical mechanism of the convulsive action of folic acid The present results support the hypothesis that folic acid interacts with the $GABA_A$-receptor complex The effect of folic acid on the binding of the cage-convulsant ^{3}H-TBOB resembles that of bicuculline and not that of βCCE This finding supports our suggestion of a common biochemical mechanism for bicuculline and folic acid Whether folic acid and bicuculline share indeed a common binding site remains to be elucidated

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In the second part of this thesis the in vitro investigations concerning the biochemical mechanism responsible for the convulsive effects of folic acid have been described

- The rank order of the potency of folates in causing epileptogenic effects was determined in vivo by quantification of the seizures following intracortical application in rats (ch 6) The rank order is

  folic acid ≥ 5-HCO-H_4folate H_2folate > 5-CH_3-H_4folate

- This sequence of epileptogenicity was compared to the rank order of the effects of folates on radioligand binding to the $GABA_A$-receptor-complex in vitro (ch 6) No correlation of the strength of the epileptogenic effects of the folates with their inhibitory potencies on ^{3}H muscimol binding (high affinity GABA site) was observed, nor with the inhibitory potencies on ^{3}H-diazepam binding (benzodiazepine site) Folates reverse the inhibiting effect of GABA on the binding of ^{3}H-TBOB The rank order of this in vitro effect

  folic acid 5-HCO-H_4folate > H_2folate ≈ 5-CH_3-H_4folate

  does correlate with the rank order of epileptogenicity determined in vivo

This result supports our hypothesis that folic acid exerts its epileptogenic action through an interaction with the $GABA_A$ receptor complex

The nature of the interaction of folic acid with the $GABA_A$ complex was investigated as well

- The inhibitory effect of GABA and the reverse effect of folic acid on ^{3}H-TBOB binding were found to result from a modulation of the apparent number of ^{3}H-TBOB binding sites rather than from a modulation of the apparent affinity of the radioligand (ch 7)
- Finally (ch 8) folic acid not only reverses the inhibitory action of GABA on ^{3}H TBOB binding but also reverses that of diazepam The GABA antagonist bicuculline mimics these actions of folic acid These observations will be further elaborated on in chapter 9 Conclusion

In het tweede deel van dit proefschrift werden de in vitro experimenten betreffende het epileptogene werkingsmechanisme van foliumzuur beschreven

- *De mate waarin de verschillende folaten epileptogene effecten kunnen induceren werd eerst in vivo bepaald door de aanvallen die optraden na intracorticale injectie van de folaten te quantificeren (hfdst 6) De rangorde is*

  folium zuur > 5-HCO H_4folaat > H_2folaat ≥ 5 CH_3 H_4folaat

- *Deze in vivo epileptogeniciteitsreeks werd vergeleken met de rangorde in vitro van effecten op de binding van radioliganden aan het $GABA_A$ receptor complex (hfdst 6) Tussen de sterkte van epileptogeniciteit in vivo enerzijds en het vermogen om de ^3H muscimol binding (hoge affiniteit GABA plaats) of de ^3H diazepam binding (benzodiazepine plaats) te verdringen anderzijds werd geen correlatie gevonden*

  Folaten bleken de verdringing van ^3H-TBOB (convulsive plaats) door GABA tegen te gaan De rangorde van dit in vitro effect

  foliumzuur 5-HCO-H_4folaat H_2folaat ≈ 5-CH_3-H_4folaat

  correleert wel met de volgorde die in vivo gevonden werd

Dit resultaat ondersteunt de hypothese dat door foliumzuur geïnduceerde epileptogene verschijnselen veroorzaakt worden door een interactie van foliumzuur met het $GABA_A$ complex

De aard van de interactie van foliumzuur met het $GABA_A$ complex werd eveneens onderzocht

De verdringing van ^3H-TBOB door GABA en het tegengestelde effect door foliumzuur bleken te komen door een verandering in het aantal receptoren zoals dat gemeten wordt en niet door een verandering in de gemeten affiniteit van ^3H TBOB voor zijn receptor (hfdst 7)

- *Tenslotte (hfdst 8) foliumzuur keert niet alleen de verdringing door GABA om maar ook die van diazepam De GABA antagonist bicuculline heeft het zelfde effect als foliumzuur op de door GABA of diazepam verdrongen ^3H-TBOB binding Op deze waarnemingen zal in de conclusie verder worden ingegaan*

In this thesis a causal relationship between the in vivo and the in vitro effects of folic acid is hypothesized We propose that the prevention of GABA induced inhibition of ^{3}H-TBOB binding is the biochemical basis of the observed epileptic phenomena However a number of questions remain to be answered

1) Is the site of interaction of folic acid with the $GABA_A$ complex the low affinity GABA site?

From a pharmacological point of view it is reasonable that a drug (folic acid) which diminishes the action of an anticonvulsive compound (GABA) might have convulsive properties We therefore think it is plausible that our hypothesis holds true (i e the prevention of GABA induced inhibition of ^{3}H-TBOB binding is the biochemical basis of the observed epileptic phenomena)

The exact point of interaction of folic acid with the $GABA_A$ receptor complex remains to be elucidated however

It is not likely that folic acid interacts with the nanomolar affinity GABA sites or with the (nanomolar affinity) benzodiazepine sites (ch 6) Another binding site must be involved GABA is thought to inhibit ^{3}H-TBOB binding through an allosteric interaction of the micromolar affinity GABA binding site with the convulsant site [15, 25, 28 ch 5] Question 1 originates from these considerations

In favour of an affirmative answer would be the following considerations

- Bicuculline is thought to bind to this low affinity GABA site [16, 21]
- When injected into the neocortex folic acid is mimicked by bicuculline (ch 1)
- Folate modulation of ^{3}H-TBOB binding in vitro is mimicked by bicuculline (ch 8)

On the other hand however

> When injected into the prepiriform cortex, folic acid does not mimic the action of bicuculline (ch 4), so an exactly similar mechanism of action is not likely

Competition studies using radiolabeled ligands with high selectivity and sufficient affinity for the $GABA_A$ low affinity binding sites (e g ^{3}H-bicuculline (K_d=40 nM) [21], or ^{3}H-SR 95531 (K_d= 8 nM) [10]) will be necessary to answer the question whether folates bind to the low affinity GABA site [16]

2) Is there a $GABA_A$ receptor heterogeneity for the interaction with either folates or bicuculline?

A variety of chemically diverse compounds has been shown to reverse GABA inhibition of ^{35}S-TBPS binding [3 5 12 22 27 28 29] analogous to our description of folate effects on ^3H-TBOB binding (ch 6) Modulatory sites on the GABA complex conducting these effects have been postulated [25]

Assuming the interaction of the folates with a modulatory site it is tempting to assume that there are regional differences in the effects of the GABA modulators, resulting e g from a different expression of $GABA_A$ receptor units [14 23] Such regional functional differences have been postulated before for the benzodiazepine sites on the complex [4 13 19 20 26] This was a great stimulus in the search for compounds with disease specific activities, lacking the global sedating properties of the classical benzodiazepines [2 6, 8 19] An investigation of the postulated regional differences of the modulatory site may be of importance in the search for disease-specific drugs for two reasons

- Many of the reported modulators of the convulsant binding site are commonly used clinical drugs (antidepressants antipsychotics, antihistaminics and compounds such as caffeine, theophylline)
- Folate deficiency is associated with a variety of neuro-psychiatric disturbances (depression psychosis and dementia [1])

3) What mechanism can explain the changes in the number of ^3H-TBOB binding sites in the presence of GABA or folic acid?

In chapter 7 we showed that folic acid reverses the GABA induced inhibition of ^3H-TBOB binding in displacement assays In saturation assays, the effect of GABA inhibition of ^3H-TBOB binding appears to be a reduction of the apparent number of binding sites (i e a decrease in R_t), whereas the effect of folic acid appears to be the reverse Question 3 arises from theoretical considerations For reversible ligand-receptor systems a change in R_t is hard to explain [11 30] A reduction in R_t can only arise from irreversible processes [31] GABA-receptor binding is not likely to be an irreversible process So, the apparent reduction of R_t may be due either to a misinterpretation of the data or to quasi irreversible processes [24]

A recent indication for the occurrence of a misinterpretation is given by Maksay and coworkers They showed that GABA and GABAergic enhancing agents ac-

celerate the dissociation rate of TBPS [17, 18] Interconvertable populations of convulsant sites with rapid and slow (dissociation) kinetics are proposed [17] An increase in the population of the rapid phase might be brought about by GABAergic enhancing drugs, and the reverse by GABAergic inhibitory compounds
In our binding assays we might have measured the high affinity sites only and may have missed the low affinity population the highest concentrations ^{3}H TBOB used were usually about 30 nM too low to measure K_d values in excess of 15 nM [9, 33] Moreover, we used the filtration technique which is generally not suitable for measuring low affinity sites [33] Saturation experiments over a wide range of radioligand concentrations (the availability of the unlabeled TBOB makes this suggestion economically feasible) combined with centrifuge techniques would be necessary to elucidate the low affinity population

4) What is the nature of the benzodiazepine interactions with ^{3}H-TBOB binding?

In chapter 8 we showed that folic acid and bicuculline reverse not only the action of GABA on ^{3}H-TBOB binding but also that of diazepam In contrast βCCE does not reverse the inhibition of ^{3}H-TBOB by diazepam The lack of interaction between βCCE and diazepam is somewhat surprising, as these ligands have opposite effects in vivo and are thought to compete for the same receptor sites [6] It is reported that β carbolines may produce their effects independently of GABA, whereas diazepam effects are GABA dependent A different coupling of the binding sites of these two substances to the Cl^--channel is proposed Moreover the concentrations of diazepam and βCCE needed to produce their effects on the binding of ^{3}H-TBOB are in excess of 100 nM These concentrations do not coincide with the reported nanomolar affinities for binding to the 'central' benzodiazepine receptor site This discrepancy pleads against an interaction of the nanomolar affinity 'central' benzodiazepine receptor sites with the ^{3}H-TBOB sites The inverse agonist, the convulsant benzodiazepine Cl-diazepam (Ro 5-4864) is reported to partially reverse the action of GABA on TBPS binding as well [7] This compound has a high affinity for the 'peripheral type' of the benzodiazepine receptors [32] Comparison of the effects of this ligand to those of the tested compounds described in this thesis may be of help in the investigation of the relationship between benzodiazepine sites and convulsant sites

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CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren op 20 november 1954 in Leiden als vijfde telg van melk- en comestibleshandelaars Wim en Annie van Rijn De middelbare school volgde zij aan de S G Bonaventura-Kijckenborg in Leiden ze behaalde in 1971 het MU LO-B examen en in 1974 het Atheneum-B examen

Daarna begon zij te studeren aan de Rijksuniversiteit in Leiden Allereerst scheikunde het kandidaatsexamen S4 (scheikunde en wiskunde met natuurkunde) werd afgelegd in 1977 tevens werd de aantekening didactiek in dat jaar behaald Tijdens deze studie ontmoette zij Jan Pieter Zwart met wie zij sindsdien het leven deelt De geneeskunde studie werd begonnen in 1977 In 1980 werd het kandidaatsdiploma hierin behaald Het 3e jaars 'keuzepracticum' organiseerde zij in Nepal Dit mondde uit in een scriptie over de 'Primary Health Care' in dat land (medeauteur I L M Rost) Het doctoraalexamen werd afgelegd in 1983 het arts diploma behaald in 1984

Vervolgens was zij verbonden aan de afdeling Neuroanatomie in Leiden waar zij o l v Dr E Marani onderzoek verrichtte aan de retina van met monosodium-glutamaat (MSG) behandelde ratten

Van oktober 1985 tot april 1987 werkte zij als promovendus op de afdeling Experimentele Neurologie in Nijmegen o l v Prof Dr O R Hommes en Dr J F Rodrigues de Miranda (afd Farmacologie) aan het in dit proefschrift be schreven onderzoek

Gedurende het jaar hieropvolgend was zij als wetenschappelijk medewerker verbonden aan het Interuniversitair Oogheelkundig Instituut (IOI) in Amsterdam waar zij kennis maakte met electronenmicroscopische technieken

Sinds april 1988 is zij als universitair docent werkzaam bij de vakgroep Vergelijkende en Fysiologische Psychologie in Nijmegen waar zij o l v Prof Dr J M H Vossen en Dr A M L Coenen onderzoek verricht aan spontane epileptische verschijnselen bij de WAG/Rij rat Tevens is zij betrokken bij het onderwijs dat door de vakgroep verzorgd wordt

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8 Peeters B W M M , Van Rijn C M Van Luijtelaar E L J M and Coenen A M L , Involvement of NMDA Receptors in Non-convulsive Epilepsy submitted

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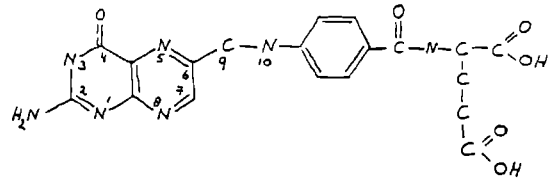
1 **FOLIC ACID**

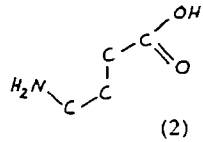


(1)

$GABA_A$ RECEPTOR COMPLEX

- GABA site

  Agonists
  2 GABA
  3 Muscimol



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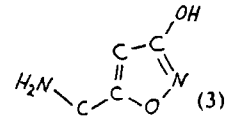
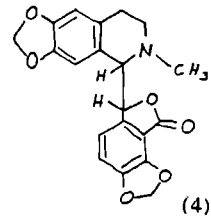


(3)

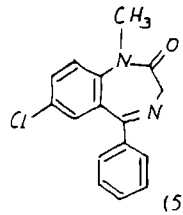
  Antagonist
  4 Bicuculline



(4)

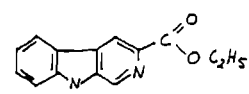
- Benzodiazepine site

  Agonist
  5 Diazepam



(5)

  Inverse agonist
  6 βCCE



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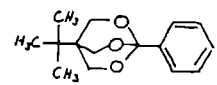
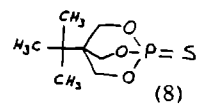
- Convulsant site

  Agonists
  7 TBOB



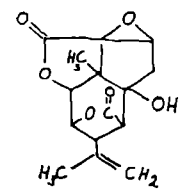
(7)

  8 TBPS



(8)

  9 Picrotoxinin

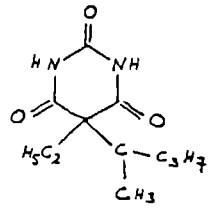


(9)

$GABA_A$ RECEPTOR (continued)

- Depressant site:

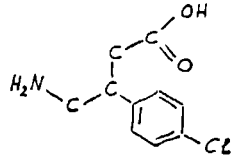
Agonist 10: Pentobarbital



(10)

$GABA_B$ RECEPTOR

Agonist 11: Baclofen



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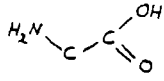
GLYCINE RECEPTOR

Agonist 12. Glycine

Antagonist 13. Strychnine



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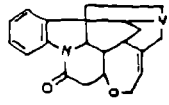
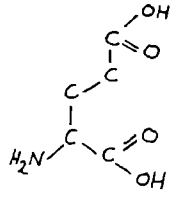


(13)

GLUTAMATE RECEPTOR

Agonists 14: Glutamic Acid

15: Kainic Acid



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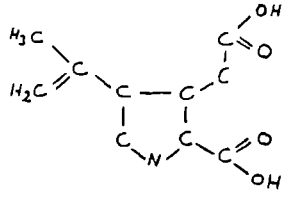

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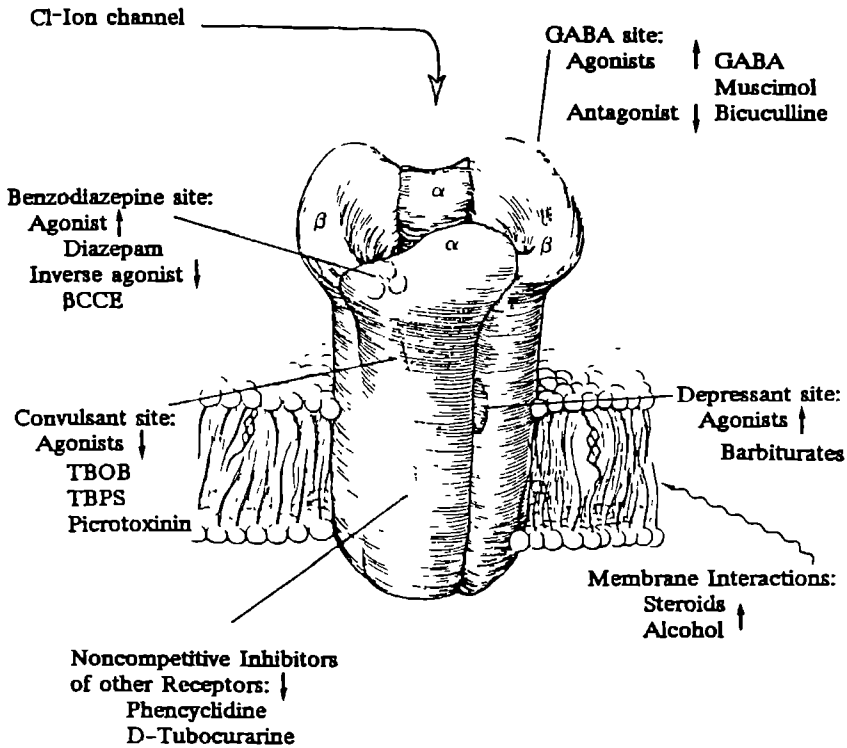


Schematic model of the GABA$_A$ complex.
Arrows indicate the enhancement (↑) or the inhibition (↓)
of GABAergic function by the various agents.

(Reproduced (modified) from R.D. Schwartz,
Biochem. Pharmacol., 37 (1988) 3396–3375, with permission)

Stellingen bij het proefschrift: Folic Acid, Epilepsy and the $GABA_A$ Complex.

1 GABA-agonisten induceren via een allostere interactie een snelle dissociatie van liganden, gebonden aan de convulsieve plaats op het $GABA_A$-complex.
- *Maksay, G., and Ticku, M.K., Life Sci., 37 (1985) 2173-2180.*
- *Dit proefschrift hfdst. 5.*

2 De waarneming, dat GABA-agonisten niet alleen de dissociatiesnelheid maar ook de associatiesnelheid beïnvloeden van liganden, die binden aan de convulsieve plaats op het $GABA_A$ complex, wijst erop dat de vooronderstelling, dat de associatiesnelheid alleen afhankelijk is van diffusieprocessen niet juist is.
- *Maksay, G., and Simonyi, M., Mol. Pharmac., 30 (1986) 321-328.*

3 Hoewel moeilijk te bewijzen is, dat de bij knaagdieren spontaan optredende piek-golf complexen epileptisch van aard zijn, kunnen dieren met deze verschijnselen goed gebruikt worden als een model voor mensen met absence epilepsie.
- *Kaplan, B.J., Exp. Neurol., 88 (1985) 425-436.*
- *Marescaux, C. et al., Epilepsia, 25 (1984) 326-331.*
- *Coenen, A.M.L. and Van Luijtelaar, E.L.J.M., Epilepsy Res., 1 (1987) 297-301.*
- *Peeters, B.W.M.M. et al., Neurosci. Res. Communications, 2 (1988) 93-97.*

4 Muscimol heeft een uitgesproken anticonvulsieve werking bij chemisch geïnduceerde focale epilepsie in de rat, ongeacht of deze convulsies geïnduceerd worden door disinhiberende stoffen of door direct exciterende stoffen.
- *Van Rijn et al. in voorbereiding.*

5 De waarneming dat potentiëring van de inhibitie bij een diermodel voor absence epilepsie het aantal piek-golf complexen per tijdseenheid doet toenemen, terwijl dit bij een diermodel voor focale, convulsieve epilepsie een antiepileptische werking heeft, doet vermoeden dat er een fundamenteel verschil bestaat tussen convulsieve en niet-convulsieve epilepsieën.
- *Myslobodsky, M.S., In R.G. Fariello et al. Neurotransmitters, Seizures and Epilepsy II, Raven Press, New York, (1984) 337-345.*

6 De observatie dat benzodiazepine-agonisten zowel convulsieve epilepsie als absence epilepsie onderdrukken doet vermoeden, dat bij de werking van benzodiazepinen meerdere receptoren betrokken zijn.
- *Schoemaker, H, et al., Eur. J. Pharmacol., 71 (1981) 173-175.*

7 Het werkingsmechanisme van een epilepsie beïnvloedend farmacum hoeft niet noodzakelijkerwijze waardevolle aanwijzingen op te leveren omtrent het mechanisme, dat ten grondslag ligt aan de spontaan optredende epilepsie.
- *Bradford, H.F. and Peterson, D.W., Molec. Aspects Med. 9 (1987) 119-172.*

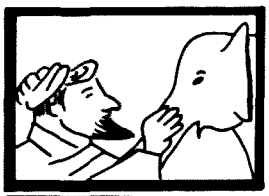
8 Om de effecten van een psycho-actieve stof te bestuderen, kan niet volstaan worden met het beoordelen van het alleen het EEG; gedragsobservaties zijn hierbij ook noodzakelijk.
- *Coenen, A.M.L. and Van Luijtelaar, E.L.J.M., Proc. WASAR-Meeting, Bazel, 1989.*

9 Het valt zeer te betreuren, dat waar sportcompetities integraal op de televisie worden uitgezonden, internationale muziekconcoursen het op dit medium veelal met een korte samenvatting moeten stellen.

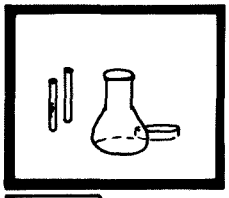
10 De hoge tempi, tegenwoordig in de mode bij uitvoeringen van barokmuziek, gaan vaak te zeer ten koste van toonvorming en expressie.

11 Het gebruik van de zinledige term 'chemische stof' kan er toe leiden, dat de schadelijke werkingen van niet als zodanig aangeduide stoffen ten onrechte onderschat worden.

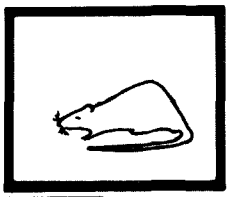
Tineke van Rijn, 22 juni 1989.



POEM



VITRO



VIVO