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1-Hydroxy-3-amino-pyrrolidone-2 (HA-966): a new GABA-like compound, with potential use in extrapyramidal diseases

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Summary

1. The drug HA-966 (1-hydroxy-3-amino-pyrrolidone-2), which chemically resembles the cyclic form of GABA, has been studied for neuro-pharmacological properties and for effects on the catecholamine content of the corpus striatum.

2. The acute effects on spontaneous behaviour of rodents included flaccid catalepsy and reversible tranquillization in doses which were 5% or less of the lethal dose. Long lasting depression of the CNS, followed by complete recovery, was produced in the cat and the dog. In the monkey HA-966 caused periodical sleeping episodes.

3. The exploratory behaviour and the amphetamine-induced motor activity in mice were blocked by HA-966. The toxicity of amphetamine in aggregated mice was only moderately reduced, suggesting that HA-966 differs from neuroleptics.

4. Tremors induced by chemical agents (nicotine, zinc and tremorine) were markedly inhibited by HA-966. The muscarinic effects of tremorine were not reduced by HA-966, indicating a selective central antitremor effect.

5. HA-966 elevated the threshold to strychnine convulsions and abolished the ipsilateral flexor reflex, while not having motor endplate blocking properties. It is suggested that HA-966 depresses central internuncial neurones.

6. In rats and rabbits HA-966 produced synchronous EEG and inhibited the sensory arousal in doses not causing sedation. In the monkey the drug caused a periodical dissociation between 'sleep-EEG' and behaviour.

7. In rat brain, HA-966 selectively elevated the dopamine content in the corpus striatum, while no changes in noradrenaline and 5-hydroxytryptamine contents could be demonstrated. The effect was still present when dopa synthesis was inhibited with α-methyl-*p*-tyrosine.

8. Several effects of intravenously administered HA-966 became manifest after an appreciable delay and in hepatectomized mice the effects were much reduced. It is postulated that HA-966 is converted to a pharmacologically active metabolite.

9. The results are discussed in the light of current views on drug therapy in extrapyramidal conditions and a GABA-related hypothesis as to the mode of action of HA-966 is presented.

Introduction

1-Hydroxy-3-amino-pyrrolidone-2 (HA-966, Fig. 1) was prepared in 1959 within the framework of a synthetic chemical programme on amino-acids (Havinga, Kerling & Roorda, unpublished) and is chemically related to the cyclic anhydric form of GABA. Initial pharmacological studies performed between 1959 and 1961 had shown that HA-966 might exert a marked influence on the extrapyramidal system, besides having other neuropharmacological effects. Pilot clinical trials showed that HA-966 appeared to benefit patients with tremors of extrapyramidal origin. Subsequent investigations at various clinical centres, however, produced some contradictory results. Our interest in the compound was again increased when, after the finding that in parkinsonian patients a gross depletion of striatal dopamine occurs (Hornykiewicz, 1963), one of us (A.W.S.), demonstrated that the compound elevates the dopamine concentration in the corpus striatum of the rat. This paper gives an account of the neuropharmacological properties and some biochemical effects of HA-966 in laboratory animals.

Methods

Animals

All experiments on mice were performed on male albino animals weighing 18–25 g. Similarly male albino animals (250–300 g) were used for experiments on rats. Some of the mice and rats were bred in this laboratory and others were purchased from TNO Animal Centre (Zeist, The Netherlands). Adult cats of either sex were used. The dog experiments were performed partly on mongrels and partly on beagles. The latter, as well as the male chinchilla rabbits (2–3 kg weight), were bred in our own laboratory. The Rhesus monkeys of either sex were purchased from a laboratory-animal forwarding agency (Heesch, The Netherlands), and were kept for several weeks in quarantine before the experiments were started. On all occasions when the drugs were administered orally, the animals were starved overnight before they were treated.

Behaviour studies

Spontaneous behaviour of various species

Groups of mice were treated intravenously and orally with a wide range of doses of HA-966. Each group received only a single dose and the mice were observed

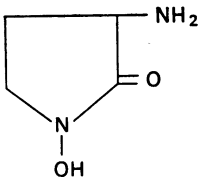


FIG. 1. Structure of 1-hydroxy-3-amino-pyrrolidone-2 (HA-966)

for any change in spontaneous behaviour, gross alteration in motor activity and maintenance of the righting reflex. Similar experiments were performed on rats. A cat received HA-966 orally by gavage. Dogs were treated either by injection of the drug into a superficial vein of the hind leg, or orally with tablets. Rabbits were injected intravenously into one of the marginal ear veins. One monkey received the drug into the cubital vein, while the others were orally dosed with tablets. All behaviour changes and neurological signs were observed on individually caged animals.

Motor activity

Hypermotility in mice was induced by suddenly placing the animals in new surroundings, where they display the so-called exploratory behaviour. The use of this type of hyperactivity for testing tranquillizing drugs is well known and has been described previously (Bonta, 1958 ; Bonta, Delver, Simons & de Vos, 1960 ; Borsy, Csányi & Lázár, 1960). In other experiments the effect of HA-966 was measured in mice which received a single intraperitoneal dose of amphetamine (5 mg/kg). The jitter cage apparatus and the statistical design applied to both types of experiments has previously been published (Bonta *et al.*, 1960).

Group toxicity of amphetamine

When crowded mice receive amphetamine, the toxicity of the latter is markedly reduced by low doses of neuroleptic drugs. The method, described by Lasagna & McCann (1957) was used. Groups of twenty mice were placed in a cage of 980 cm^2 surface and an intraperitoneal dose of amphetamine (12·5 mg/kg) was administered. The animals were observed for 5 h and the number of dead mice was recorded.

Chemically induced tremors

Mice were used for all these experiments. The use of tremors induced by nicotine in rabbits was suggested by Bovet & Longo (1951) to investigate antiparkinsonian drugs. Subsequently Stone, Meckelnburg & Torchiana (1958) showed that the clonic seizures induced in mice by the high dose of nicotine (0·84 mg/kg) were resistant to antiparkinsonian agents. In the study described here nicotine (0·240 mg/kg, i.v.) was used for tremor induction. The short duration of these tremors made objective visual evaluation hardly possible, hence we used instrumental recording, as described previously (Bonta *et al.*, 1960). The mice were randomly divided into groups of ten. Separate groups were used for saline treated controls and those receiving drugs, which were intraperitoneally administered 30 min before nicotine injection. The percentage difference between the intensity of tremor of saline and drug treated groups was used as the criterion of tremor inhibition.

Tremorine (1;4-dipyrrolidinobut-2-yne) provides a tool to show whether there is a correlation between antitremor and antimuscarinic activity of an antiparkinsonian drug. Randomized groups of ten mice were treated intraperitoneally with tremorine (20 mg/kg). The occurrence of tremors and muscarinic symptoms (salivation, lachrymation, defaecation) in individually caged mice was visually observed 60 min after the tremorine. Saline or the drugs were administered intraperitoneally 15 min before tremorine. The number of positive reactors in each group was

counted and inhibition of tremor or muscarinic symptoms was expressed as the percentage difference between saline and drug treated groups.

Administration of zinc acetate into the third brain ventricle in mice induces a dyskinetic syndrome consisting of intermittent hind limb tremors, running fits, rigid gait, backward movements and convulsions (Bonta, van der Burgh & Greven, 1964). With a suitable dose of zinc acetate, yielding after dissociation 0·09–0·12 mg/kg Zn^{++} (ionized zinc), approximately 70% of the mice display the characteristic hind limb tremors, while the other symptoms occur less regularly. The tremor-inhibiting effect of the drugs was observed on mice which received an equivalent of 0·09 mg/kg Zn^{++} by the intracerebral injection technique of Haley & McCormick (1957).

Protection against strychnine

The intravenous titration method of Jenney & Pfeiffer (1956) was used. A 0.005% solution of strychnine was injected at the rate of 0·05 ml every 10 s into the tail vein of mice. The amount which caused death of the animals was recorded. The incidence of tonic extensor thrust before death was also noted. HA-966 was administered intraperitoneally 20 min before strychnine.

Spinal reflexes

A total of twenty-six cats were used, twenty spinalized and six intact. For spinalization the animals were anaesthetized with ether, a tracheal cannula inserted and the spinal cord transected at the atlanto-occipital junction. Artificial respiration was given with a Palmer pump and the anaesthesia was discontinued. Two–three hours were allowed for elimination of the ether before the actual experiment was commenced. The intact cats were anaesthetized with an intraperitoneal dose of 2 ml/kg of a solution containing chloralose, 5% and urethane, 25%, a tracheal cannula inserted and artificial respiration applied throughout the whole experiment. The ipsilateral flexor reflex was evoked by stimulating the central trunk of the transected N. tibialis and the contractions of the tibialis anterior muscle were recorded. In several experiments the peripheral trunk of the transected N. peroneus communis of the contralateral leg was also stimulated and the contractions of the M. tibialis anterior recorded. Square wave stimulators, which were automatically triggered through an electric time clock, served for firing repeated stimuli at regular intervals. The stimuli for the nerve-muscle preparation were of 1 ms duration and those for the ipsilateral flexor reflex of 10–20 ms duration. The strength of the stimuli varied between 5 and 10 V depending on the individual sensitivity of the cats ; however within any one experiment the strength was kept constant. In some of the experiments intervals of 2 min were used and in other intervals of 30 seconds. In one series of experiments contractions were recorded by attaching the distal tendon of the muscle to a mechanical isometric lever which was writing on a smoked kymograph drum. In the other series the muscle tendon was attached to a force-displacement transducer (FT-10) and a Grass Model-7 four channel polygraph was used for recording. In the experiments on non-spinalized cats the blood pressure was recorded from a carotid artery which was connected to a Statham pressure transducer. Drugs were injected through a polythene cannula into a jugular vein.

Electroencephalographic studies

Rat experiments

A total of twenty-three rats were used for EEG studies. The technique for implanting electrodes and recording cortical electrograms was described earlier (de Vos & Bonta, 1964). After the surgical procedure a rest of 2 days was allowed for the animals in which acoustic activation of EEG was studied. After a control tracing was taken and the rats displayed a quiet EEG pattern, a buzzer was sounded for 2 seconds. The activation of the EEG in four leads (left-right frontal, left-right parietal, left fronto-parietal, right fronto-parietal) was measured for duration, averaged and expressed in seconds. The acoustic stimulus was applied for 2 s, the response, however, lasted 9–60 s in 85% of the rats and in the rest even longer. In the latter case, for practical reasons, 60 s was considered as a maximal response. The stimulus was repeated 10, 30, 60, 120, 150 and 180 min after intravenous administration of the drug. For each time interval the average effect for each dose was obtained from four experiments. A group of fifteen rats served as saline treated controls. They were randomly divided over the whole period during which this series of experiments was performed. HA-966 was administered into the tail vein. During the EEG recording the rats were freely moving in an animal acoustic chamber (Type Model AC-3, IAC Inc.) and two rats were traced simultaneously. The EEG was traced on an eight-channel pen recorder amplifier (Type ES 8, van Gogh), which was outside the cabin.

Rabbit experiments

The experiments were carried out on twenty-four rabbits, having an average weight of 2·65 kg. The stereo taxic system of Monnier & Gangloff (1961) was used for the implantation of the recording and stimulating electrodes. Placement of the electrodes was performed under local anaesthesia and the experiment was carried out a few hours later. The sound of a buzzer for 2 s served as the acoustic stimulus. In order to prevent habituation, the stimulus was applied at intervals not shorter than 20 minutes. Activation of the EEG (low voltage—fast wave) in the sensomotor cortex served as response and the duration of this activation time was considered as 100% response. In 40% of the rabbits the activation lasted 6–10 s and in 60% more than 10 seconds. The latter however was chosen arbitrarily as a limit to be considered as a maximal response. After having obtained a control response, the drug was administered into the marginal ear vein and the stimulus repeatedly applied. The arousal duration was compared with the control response and expressed in percentage terms. The mesencephalic reticular formation was stimulated through platinum electrodes which were connected to a Grass Model-S4 stimulator, the latter being provided with a SIU-4 stimulus isolation unit. The stimulus duration was 4 s and consisted of trains of biphasic rectangular pulses at a frequency of 200 Hz and a duration of 0·4 milliseconds. The strength of the stimulus was of threshold character to obtain clear activation of the EEG in the sensomotor cortex. The first determination was performed in the predrug period, thus each rabbit serving as its own control. Determination was repeated at regular intervals not shorter than 20 minutes. The drug was administered intravenously as a single dose. During all experiments the rabbits were fixed in a hammock similar to that described by Monnier & Gangloff (1961). Conditions for keeping the animals in a cabin and recording the EEG were similar to those described above for rats.

Monkey experiments

A male Rhesus monkey of 2·5 kg was trained to sit in a specially designed restrain chair. Cortical screw electrodes were implanted in the skull (bilateral frontal and bilateral parietal) under nitrous oxide-fluothane anaesthesia. A separate grounding electrode was placed on the sagittal suture behind the frontal electrodes. During the EEG tracing the monkey in the chair was placed in a Faraday cage, outside which was the eight channel amplifier. Six weeks after implantation of the electrode an experiment was performed with HA-966, which was orally administered by a stomach tube. The experiment was repeated several months later.

Hepatectomized mice

Under light ether anaesthesia laparotomy was performed in mice and two-thirds of the liver was removed. Sham operated mice (laparotomy but no hepatectomy) served as controls. The central muscle relaxant effect of HA-966 monitored by loss of grip strength was tested 1 h after the operation. The mice were placed on a vertical wire mesh, on which they stay normally for several minutes, but animals with relaxed muscles are incapable of supporting themselves and slide down. The end-point of the assay was sliding down within 5 seconds.

In one series of experiments the mice were treated with carbon tetrachloride (5% solution, 0·2 ml/mouse) 48 h before subtotal hepatectomy, thus ensuring that any remnants of the liver were not functioning normally.

Determination of monoamines in rat brain tissue

The rats were decapitated and the brains, with the exception of the olfactory bulbs and cerebella, were rapidly removed. The corpora striata were dissected, placed into ice cold perchloric acid (0·4 N) and homogenized with a ' Silverson ' metal homogenizer. Monoamines were analysed fluorimetrically after cation-exchange chromatography. Concentrations of dopamine (DA) were determined by the method of Carlsson & Waldeck (1958) with the modifications described by Carlsson & Lindquist (1962), noradrenaline (NA) according to the procedure of Bertler, Carlsson & Rosengren (1958) and 5-hydroxytryptamine (5-HT) by the method of Anden & Magnusson (1967). Additionally COMT activity was measured by a colorimetric assay (Anderson & D'Iorio, 1966) using rat liver supernatant as enzyme source.

Drugs

The following drugs were used: atropine sulphate, caramiphen hydrochloride (panparnit), diethazine hydrochloride (diparcol), (±)-amphetamine sulphate, strychnine nitrate, 1,4-dipyrrolidinobut-2-yne (tremorine), nicotinic acid tartrate, 1-hydroxy-3-amino-pyrrolidone-2 (HA-966), (Organon N.V.), DL-α-methyl-*p*-tyrosine methyl-ester hydrochloride (Sigma).

Before use the drugs were dissolved in 0·9% NaCl solution. The doses mentioned in the text refer to the salts. Tablets were prepared at the Pharmacy Department of N.V. Organon (Oss, The Netherlands).

Results

Spontaneous behaviour

Rodents

When HA-966 was given intravenously to mice in a dose of 6 mg/kg, a decrease of spontaneous activity was seen after a latent period of 15–20 min and the same delay was observed before the onset of flaccid catalepsy induced by 25–50 mg/kg. The animals remained passively in unnatural positions, but the righting reflex was maintained. The cataleptic state lasted for about 90 minutes. Doses of 400 mg/kg or higher abolished the righting reflex ; however, complete recovery took place after 5–6 h even with the dose as high as 800 mg/kg. A dose of 1,600 mg/kg killed two out of six mice, while in the surviving four mice no behavioural abnormality was observed after their recovery from this huge dose. It should be emphasized that the intravenous administrations of HA-966 was invariably followed by a latent period, though with the higher doses this was not longer than 4–5 minutes. The effects of orally administered HA-966 were similar to those obtained by intravenous injection, except that the onset of action was slightly delayed (Fig. 2). Decreased motor activity after oral administration could be observed after a dose of 25 mg/kg, while even 2,000 mg/kg killed only one out of sixteen mice within the observation period of 48 hours. In rats and rabbits qualitatively similar effects were obtained as in mice. The doses required to produce behavioural alterations are compiled in Table 1 which shows that the margin between the dose required to decrease motility and the lethal dose is greater in mice than in the other two species.

Cat

Twenty minutes after the oral administration of HA-966 (100 mg/kg) the spontaneous activity of the cat entirely disappeared. Thirty minutes later flaccid paralysis of the hind legs associated with a spastic rigidity of the forelimbs was observed. One hour after the drug the cat was in complete flaccid paralysis, with maintained corneal and knee jerk reflex. The pupils were rather myotic. The animal remained in this state for 7 h, after which gradual recovery was seen and by 15 h after the drug, the cat was apparently normal.

Dogs

Three animals received the drug intravenously (50 mg/kg). The symptoms were rather similar in all three animals: after a latent period of 10–15 min salivation appeared and the dogs became sluggish. Salivation was extremely marked for several hours and two of the three dogs vomited. Twenty minutes after the injection progressive muscular weakness set in and 1 h after drug administration the dogs were lying on their sides. They appeared, however, to react to external stimuli and the impression was gained that they were not unconscious but somatically paralysed. The animals remained in this state for several hours, after which complete recovery took place.

Three other dogs were given 12 mg/kg orally. One hour after ingestion of the tablets salivation, drowsiness, vomiting and muscular weakness were observed. The effects lasted for 2 hours.

Monkeys

One animal received 20 mg/kg HA-966 intravenously. After a latent period of 30 min the movements of the normally lively animal became progressively more sluggish. One hour after drug administration the monkey suddenly fell asleep in its sitting posture. After about 5 min it awoke (Fig. 3), and similar sleeping episodes returned periodically for 2·5 hours. The intervals between two sleeping episodes varied between 5 and 20 min and the sleeping episodes themselves never lasted longer than a few minutes and could be interrupted by external stimuli. The monkey did not give the impression of a narcotized animal, but rather seemed to be 'dozing' in a natural way. Three hours after the injection the behaviour of the monkey was entirely normal and no sign of the drowsiness remained. A second monkey was treated with 100 mg/kg orally. Two hours later this animal also became sluggish and from time to time fell asleep (eyes closed). Complete recovery followed after about 6 hours.

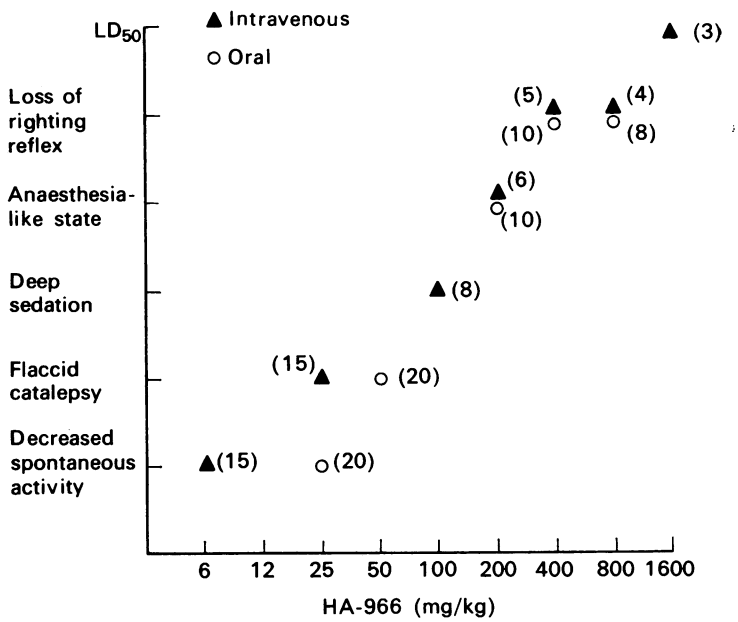


FIG. 2. Dose-effect correlation with HA-966 on spontaneous behaviour of mice. Figures in brackets indicate onset of action in minutes. Each point was obtained from five to fifteen mice.

TABLE 1. *Approximate doses of HA-966 required to cause behavioural alterations and death in rodents*

Species and route of administration		Decrease of spontaneous motility (mg/kg)	Loss of righting reflex (mg/kg)	LD50 (mg/kg)
Mouse	i.v.	6	400	1600
	p.o.	25	800	>2000
Rat	p.o.	20	150	450
Rabbit	i.v.	20	100	450

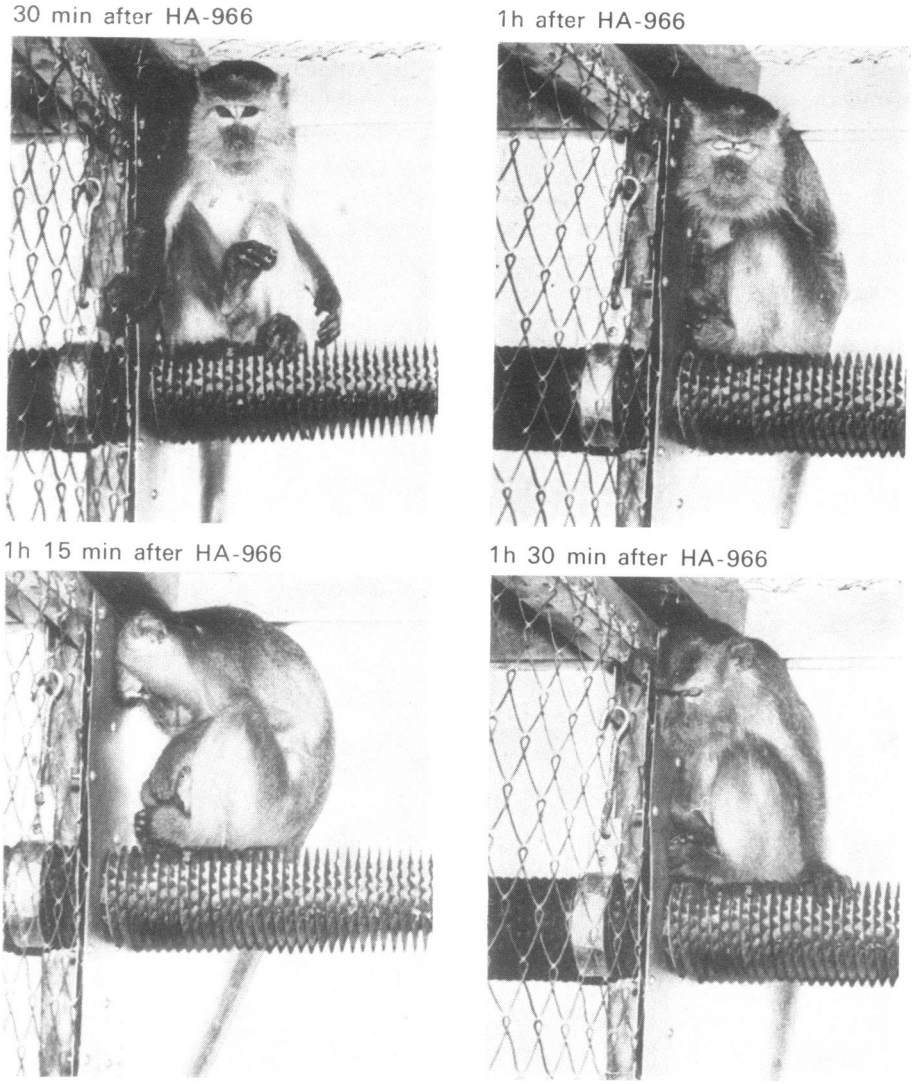


FIG. 3. Behaviour of a monkey after HA-966 (20 mg/kg i.v.) Upper left: 30 min after treatment, monkey is sluggish, but awake (eyes open). Upper right: 1 h after drug, monkey suddenly falls asleep, eyes closed. Lower left: 1 h 15 min, monkey spontaneously opens eyes. Lower right: 1 h 30 min, suddenly falls asleep again, eyes closed. Three hours after the injection complete recovery was observed.

TABLE 2. *Effect of HA-966 on the exploratory hypermotility of mice*

Route of administration	Dose of HA-966 (mg/kg)	Percent inhibition with 95% confidence limits
Subcutaneous	5	46 (25– 68)
	25	81 (59–107)
Oral	2	10 (−19– 35)
	10	77 (51–110)

Four groups of eight mice each were used at each dose level. HA-966 was administered 1 h before starting the experiment.

Effect on motor activity

As shown in Table 2, HA-966 markedly inhibited the exploratory hypermotility in mice, irrespective of the route of administration. Hypermotility induced by amphetamine was also markedly inhibited by HA-966 at doses of 8–18 mg/kg. The toxicity of amphetamine in aggregated mice was hardly reduced, however, even with doses as high as 100–200 mg/kg of HA-966.

Tremor inhibition

Tremors induced by nicotine were more effectively prevented by HA-966 than by diethazine, caramiphene or atropine (Fig. 4 upper panel). The same was true for the tremors induced by intracerebrally administered zinc (Fig. 4 lower panel). Atropine and caramiphene however prevented the tremorine induced tremors in lower doses than did HA-966, the effectiveness of the latter being comparable to that of diethazine. However, whereas atropine, caramiphene and diethazine inhibited

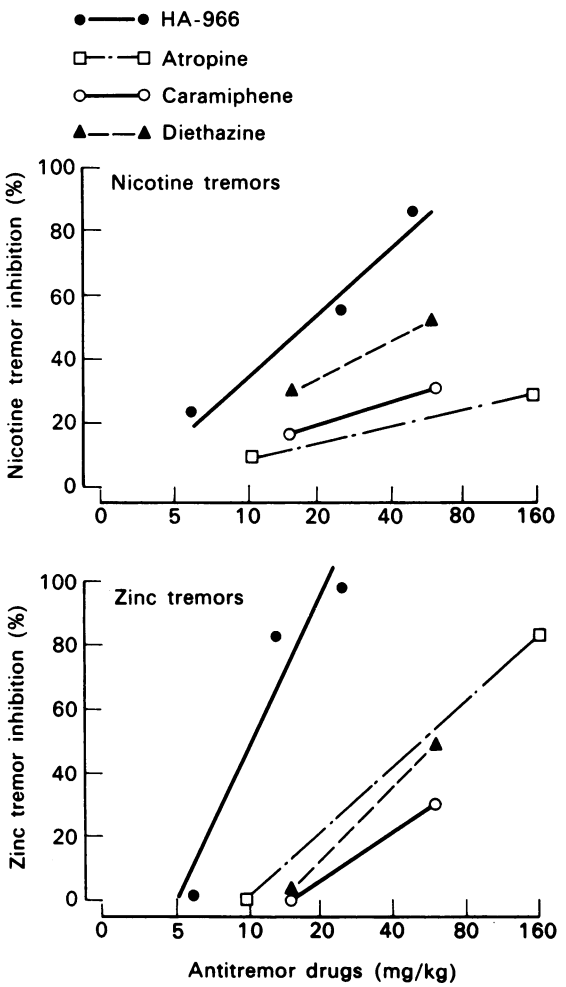


FIG. 4. Inhibition of tremors induced with intravenously administered nicotine and intracerebrally administered zinc in mice. Each point was obtained from ten mice. Further technical details are described in the **Methods** section.

both the tremors and the peripheral muscarinic effects of tremorine, HA-966 selectively prevented the tremors without influencing the muscarinic symptoms (Table 3).

Central muscle relaxant effect

HA-966 counteracted the effect of strychnine, as demonstrated in the intravenous titration assay, in which the threshold to strychnine was markedly elevated. The tonic extensor seizures were either modified to clonus or with higher doses of

TABLE 3. *Inhibition of tremorine effect in mice*

Drug	Dose i.p. (mg/kg)	Tremors Incidence	Tremors % inhibition	Muscarinic symptoms Incidence	Muscarinic symptoms % inhibition
Saline	—	55/55	—	55/55	—
HA-966	12·5	5/10	50	10/10	0
	25	2/10	80	10/10	0
	50	0/10	100	10/10	0
Atropine	5	0/10	100	0/10	100
Caramiphene	15	0/10	100	0/10	100
Diethazine	15	5/10	50	8/10	20
	60	0/10	100	3/10	70

Saline or the drugs were administered 15 min before tremorine. The latter was administered intraperitoneally in a dose 20 mg/kg. The saline treated controls were randomly divided over the period during which this series of experiments was performed.

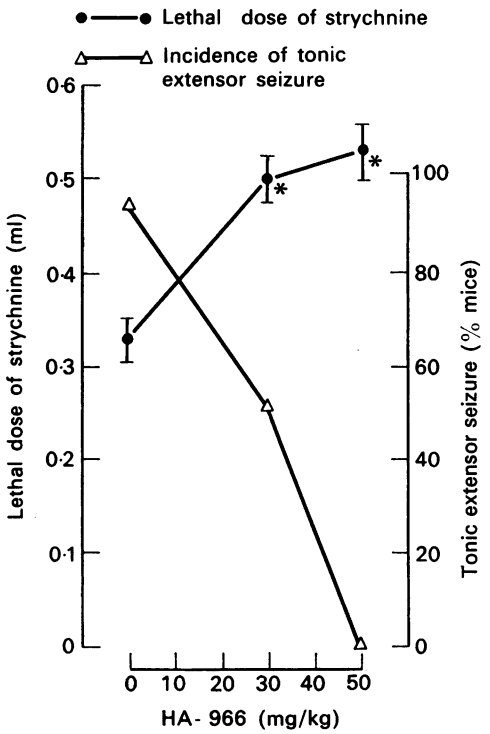


FIG. 5. Effect of HA-966 in the strychnine titration assay. HA-966 was administered intraperitoneally 20 min before the intravenous infusion of strychnine. Each point is a mean ± S.E.M. as obtained from sixteen mice. *$P<0.05$ compared to controls as calculated with Wilcoxon's rank-sum test.

HA-966 were completely abolished and such animals died of strychnine without convulsions (Fig. 5).

The ipsilateral flexor reflex was markedly inhibited or completely blocked by HA-966 in doses ranging from 5 to 15 mg/kg. Figure 6 shows an experiment in which, despite intravenous administration, there was a delay of up to 6 min before the onset of inhibition. In some experiments, however, the delay was shorter or occasionally absent. Simultaneously with inhibiting the flexor reflex, HA-966 did not reduce and occasionally even facilitated the response of the muscle to direct stimulation of the motor nerve. Such an experiment is shown in Fig. 7 from which it is also clear that the effect of HA-966 on the flexor reflex was reversed by strychnine. Clear indications were thus found that the muscle relaxation induced by HA-966 is not due to an effect on the neuromuscular junction but is centrally mediated, probably through depressing the interneurones which are involved in the polysynaptic reflex arch.

Electroencephalographic effects

Rat experiments

A single intravenous dose of HA-966 (1·25 mg/kg) inhibited the arousal measured 60 min after the drug administration. With the dose of 5 mg/kg marked inhibition

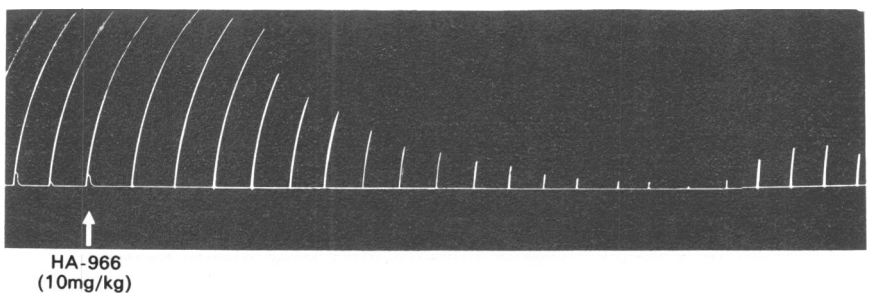


FIG. 6. Ipsilateral flexor reflex of the spinalized cat. There was an interval of 2 min between each stimulus. Note the delay of 6 min before onset of the effect of HA-966.

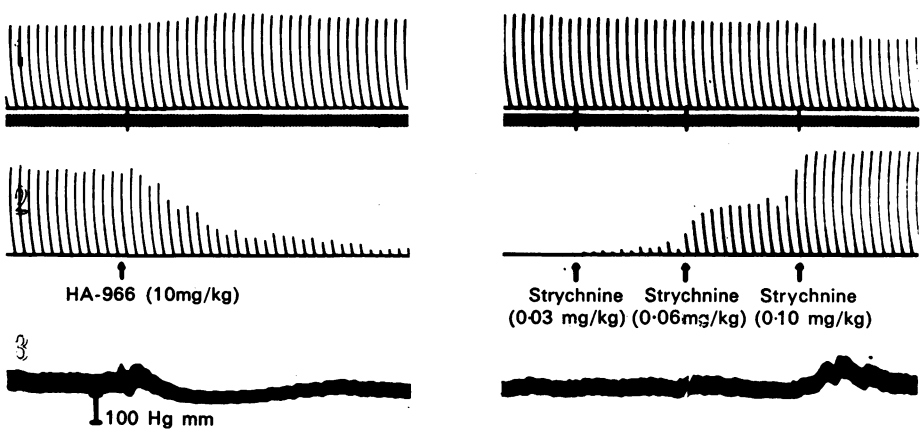


FIG. 7. Nerve-muscle preparation and ipsilateral flexor reflex of the non-spinalized cat, anaesthetized with chloralose-urethane. Tracings from top to bottom: nerve-muscle preparation, time marker 10 s, ipsilateral flexor reflex, blood pressure.

of the arousal response was observed at 30 min, but the peak of inhibition was reached at 60 minutes. It is relevant to mention that the dose of 5 mg/kg caused neither sedation of the rats nor any appreciable change in the spontaneous EEG pattern. A synchronous EEG was, however, obtained with 10 mg/kg.

Rabbit experiments

Some 10–15 min after the intravenous administration of HA-966 (10 mg/kg) a high voltage–low frequency (8–12 cycles/s) pattern appeared in the cortical leads in four out of six rabbits. This pattern was regularly interrupted by spontaneous arousals. This pattern persisted for more than 3 hours. In two rabbits the onset of the effect was one hour after the drug administration. Similar effects in the spontaneous EEG were observed with 20 mg/kg. In four out of six rabbits the onset was 10 min, and in two other rabbits 30 min after the injection.

Significant inhibition of the arousal was not observed earlier than 90 min after 10 mg/kg and 30 min after 20 mg/kg. The electrical threshold to activate the EEG by direct stimulation of the brain stem reticular system remained unchanged after 10 mg/kg and was slightly, but not statistically significantly, elevated when 20 mg/kg was administered. The doses quoted above did not cause sedated behaviour except that 20 mg/kg reduced the spontaneous motility when tested on unrestrained rabbits.

Monkey experiments

The EEG changes after oral administration of HA-966 (40 mg/kg) appeared after about 20 minutes. The characteristic changes—consisting of high voltage and very slow waves—are shown on Fig. 8. These regular slow waves were more pronounced in the parietal than in the frontal or occipital leads. Arousal response, elicited by a person entering the laboratory, was never totally blocked during the whole experimental session of 3 hours. Eighty minutes after drug administration the monkey suddenly fell asleep, but could easily be awakened. These sleeping episodes—never lasted longer than 2 min—could readily be interrupted by external stimuli. During the sleeping episodes the EEG pattern occasionally changed from high voltage–slow wave to low voltage–fast wave. In fact, the EEG during the sleep episodes resembled the arousal picture obtained in the control period before the drug. A gross dissociation between behaviour and EEG is also shown by the fact that when the monkey spontaneously opened its eyes, the fast wave activity suddenly changed into the characteristic picture of very slow wave trains in the parietal leads. Events of a periodical dissociation (shift) between EEG and behaviour are also shown in Fig. 9, which demonstrates a characteristic ' sleep EEG ', while the monkey was not asleep. Very similar results were obtained in a second experiment, performed several months later on the same animal, when the same dose of HA-966 was administered.

Effect on hepatectomized mice

Since it was observed that several effects of HA-966 became manifest or reached their peak after an appreciable delay, attention was paid to the possibility that not the compound as such, but a metabolite may possibly be the pharmacologically active material. An attempt was thus made to investigate the role of the liver in

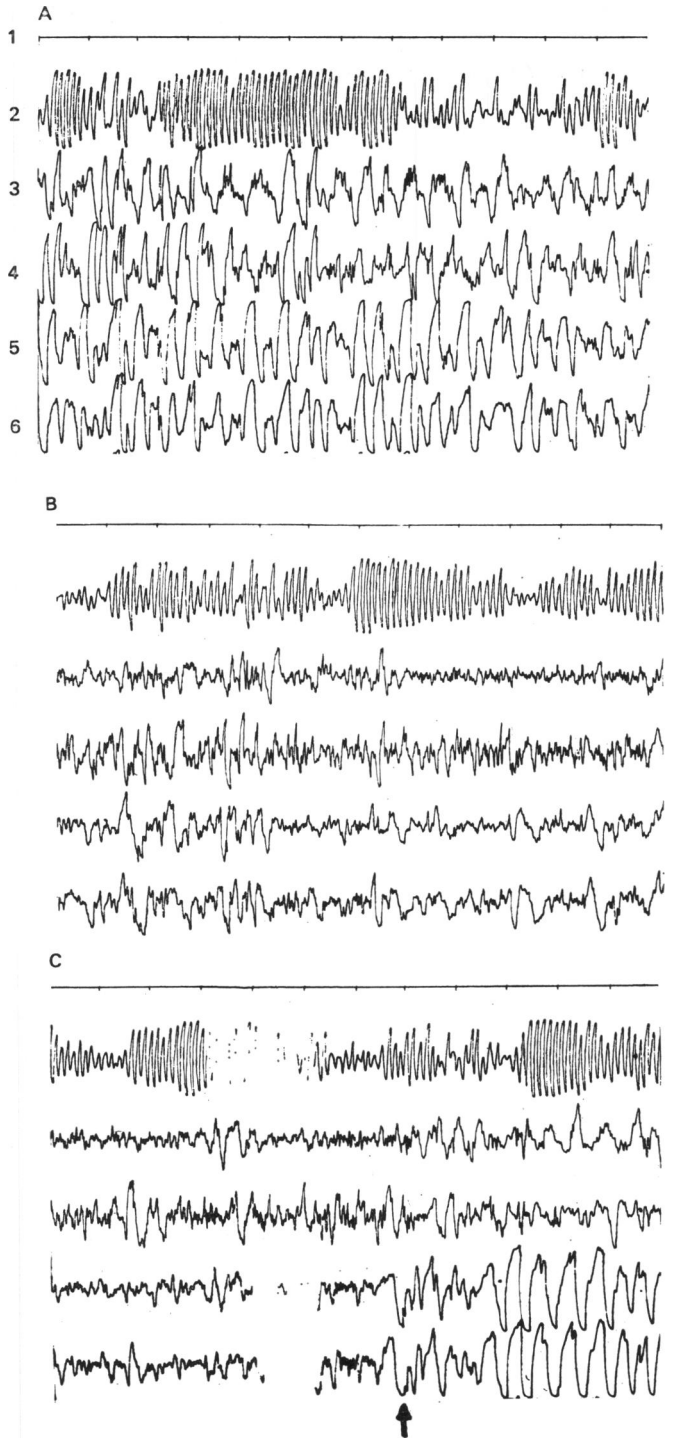


FIG. 8. EEG during sleeping episode of monkey under influence of HA-966. Tracings: (1) time marker 1 s, (2) wave analyser 8,2 Hz (frontal left-frontal right), (3) lead frontal left-frontal right, (4) parietal left-parietal right, (5) frontal left-parietal left, (6) frontal right-parietal right. Chart speed 1·5 cm/second. The recordings were made 160 min after oral administration of HA-966 (40 mg/kg). Panel (A): 20 s after the monkey closed its eyes. Panel (B): the start of this record was 33 s after (A). Panel (C): the start of this record was taken 16 s after (B). At the arrow (↑) the monkey opened its eyes spontaneously. The total duration of this sleeping episode was 100 seconds. A control tracing from the monkey in the predrug period is shown on Fig. 9.

the postulated metabolical conversion of HA-966. The loss of grip strength served as a measure of the central muscle relaxant effect in normal and hepatectomized mice. HA-966 (50 mg/kg) administered intravenously, caused a loss of grip strength in 80–100% of intact or sham operated mice, and in only 30% of mice from which two-thirds of the liver was removed. There was a slight reduction, and some delay in onset of action, in mice which were not hepatectomized but which were poisoned with carbon tetrachloride. The effect of HA-966 was, however, entirely abolished in

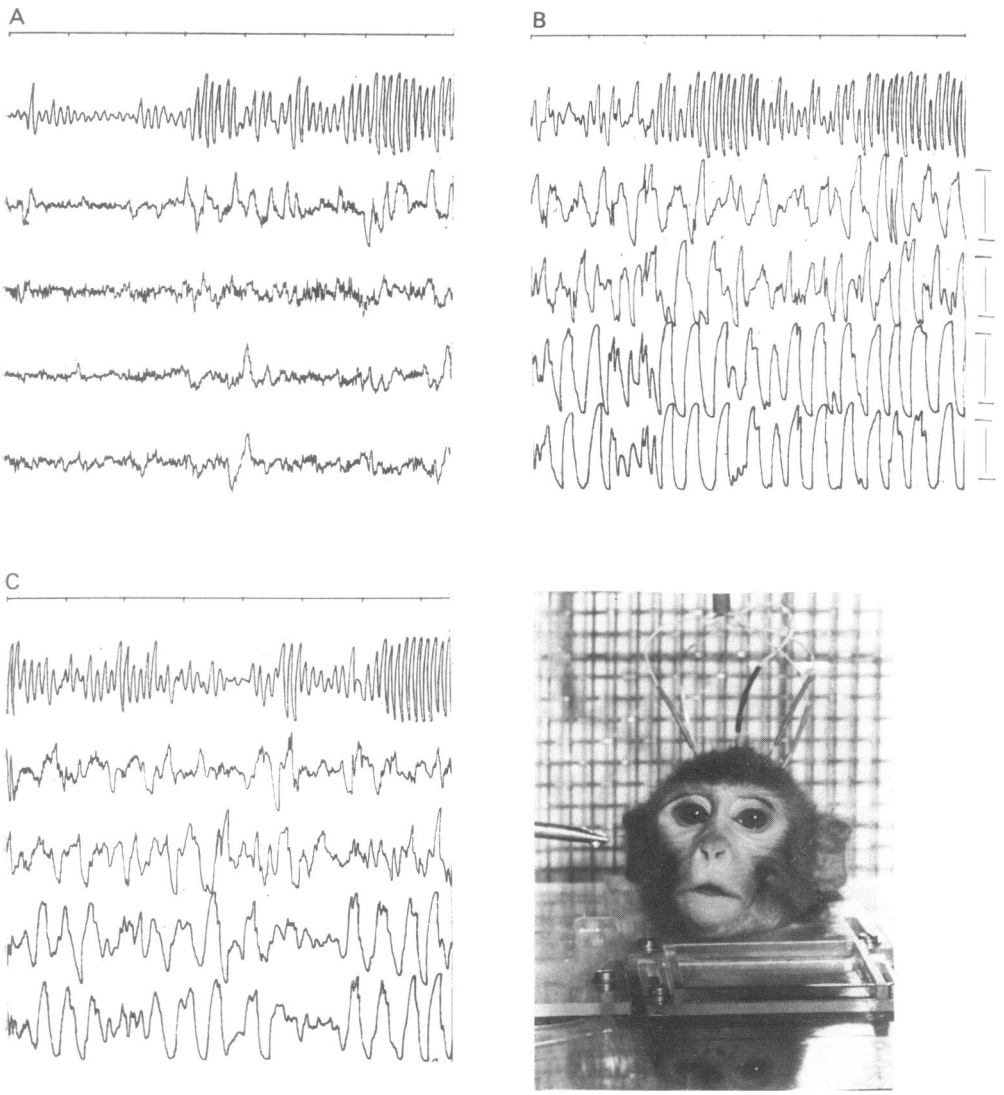


FIG. 9. Dissociation between EEG and behaviour of monkey after HA-966. Tracings from top to bottom are described in legend to Fig. 8. Calibration 200 μV. Panel (A): control record in predrug period. Panel (B): 30 min after oral administration of HA-966 (40 mg/kg). Panel (C): 82 min after HA-966. Picture of monkey was taken simultaneously with record shown on panel (C). Note that the monkey was fully awake, though EEG pattern showed high voltage-slow wave activity.

mice which were poisoned with carbon tetrachloride and additionally subjected to subtotal hepatectomy (Fig. 10).

Effect of brain monoamines

Preliminary experiments had indicated that HA-966 did not influence catecholamine concentrations if measured in the whole brain, 4 h after intraperitoneal injection (100 mg/kg) and even after chronic treatment (200 mg/kg daily, 6 days). However, in the corpus striatum, there was a marked effect on DA concentrations, while

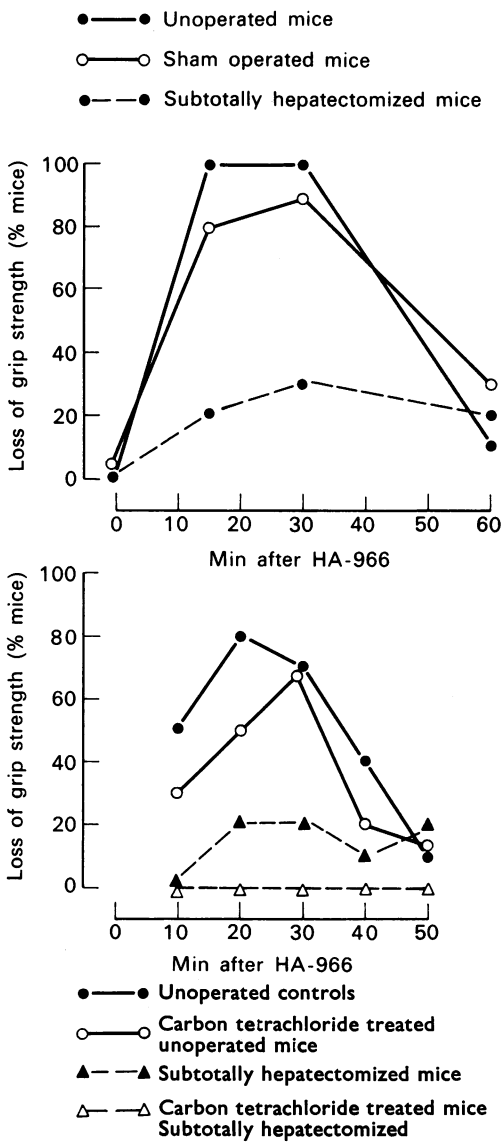


FIG. 10. Effect of HA-966 on hepatectomized mice. Each group received a single intravenous dose of 50 mg/kg HA-966. Each point was obtained from ten mice. Technical details are described in the **Methods** section.

those of NA and 5-HT were unaffected (Table 4). After HA-966 (100 mg/kg) the DA content was increased for at least 20 h as can be seen from Table 5. After short delay this dose of HA-966 produced a marked depression of the CNS, resembling anaesthesia. The occurrence of this depression did not seem to be related to the elevated DA concentrations in the striatum, since recovery of the CNS depression was almost complete after 4 h, while DA concentrations were still increased. In order to investigate the mechanism by which HA-966 raises DA concentrations, rats were given α-methyl-*p*-tyrosine (α-MPT) to inhibit the enzyme tyrosine hydroxylase, the rate limiting factor in catecholamine biosynthesis (Spector, Sjoerdsma & Udenfriend, 1965). Four hours after α-MPT (200 mg/kg i.p.), there was a decrease in the DA content to 46% of its control value. Combined treatment with

TABLE 4. *Effect of HA-966 on the concentrations of dopamine (DA), noradrenaline (NA) and 5-hydroxytryptamine (5-HT) in the corpus striatum*

	Monoamines (μg/g wet weight) Mean ± S.E.M.		
	DA	NA	5-HT
Control (saline	3·06±0·28 (9)	0·31±0·07 (9)	0·22; 0·21 ø
HA-966 50 mg/kg	4·36±0·28 (7)*	0·39±0·18 (3)	
100 mg/kg	4·76±0·41 (9)*	0·37±0·07 (9)	0·20; 0·23 ø
200 mg/kg	5·32±0·67 (9)*	0·33±0·03 (3)	

Each analysis was performed 4 h after intraperitoneal injection of HA-966. ø, Each determination was done on five pooled brains. * $P<0·05$, compared to control, Student's *t* test.

TABLE 5. *Time course of increased DA content of the corpus striatum after HA-966*

Time (h)	Dopamine (μg/g wet weight) Mean ± S.E.M.	
	HA-966	Controls
1	7·55±0·28 (5)*	3·02±0·16 (3)
4	4·76±0·41 (9)*	2·93±0·49 (3)
20	4·94±0·20 (5)*	3·21±0·80 (3)

Each analysis was performed after intraperitoneal injection of HA-966 (100 mg/kg). * $P<0·05$, compared to control, Student's *t* test.

TABLE 6. *Effect of HA-966 on catecholamine depletion by α-methyl-p-tyrosine*

	Monoamines (μg/g wet weight) Mean ± S.E.M.	
	DA	NA
Control (saline)	3·46±0·48 (5)	0·17±0·03 (6)
α-MPT (200 mg/kg)	1·60±0·19 (5)*	0·20±0·03 (5)
α-MPT (200 mg/kg)+HA-966 (100 mg/kg)	2·67±0·22 (5)*	0·27±0·02 (5)

Each analysis was performed 4 h after drug treatment. * $P<0·05$, Student's *t* test.

HA-966 markedly counteracted this depletion. Again no effects on NA concentrations could be found (Table 6). In addition, COMT activity remained unaffected by HA-966 (2×10^{-3}M) in an *in vitro* assay.

Discussion

The results show that HA-966 exerts a combination of effects, characterized by complex behavioural changes, marked antitremor activity, polysynaptic reflex inhibition and selective neurochemical alterations. The unusually wide margin between lethal doses and those causing striking behavioural changes makes it similar in these respects to major tranquillizers such as phenothiazines or certain butyrophenones.

Another similarity is the observation that HA-966 markedly prevented sensory arousal, while not causing appreciable elevation of the threshold to direct electrical stimuli on the brain stem reticular formation. The latter combination of effects indicates that HA-966 presumably interrupts the connexion between afferent sensory pathways and the reticular formation itself. Unlike major tranquillizers and hypnosedatives, HA-966 was unable to counteract markedly the toxicity of amphetamine in crowded mice. Also there are other arguments against classifying HA-966 as a diffusely acting hypnosedative drug. First, because such drugs, for example, barbiturates, impair equally both sensory and direct stimulation of the reticular formation. Second, because the changes caused by HA-966 on spontaneous EEG, particularly as observed in the monkey, are different from the ' burst-slow wave ' patterns caused by barbiturates and also by atropine (Wikler, 1952). Though short lasting periods of apparent sleep were produced by HA-966, the drug seemed to cause a shift between ' sleep-EEG ' and depression of behavioural alertness. Such a dissociation is unusual for hypnosedatives, but resembles somewhat the effect first described for anticholinergic drugs used for the treatment of extrapyramidal conditions (Rinaldi & Himwich, 1955 ; Longo, 1956).

A conspicuous property of HA-966 is its ability to counteract chemically induced tremors. A comparison of HA-966 with some recognized antiparkinsonian drugs showed that different degrees of protection against three types of tremorogenic agents was achieved. Against the subtle tremors induced by nicotine and the hind limb tremors caused by intracerebrally administered zinc, HA-966 were more potent than any of the three other antiparkinsonian drugs, while the reverse situation was true for tremors produced by tremorine. It was, however, observed with tremorine that —in contrast to the other drugs—HA-966 counteracted the tremors only, but did not inhibit to any extent the peripheral cholinergic component of the tremorine syndrome. This observation suggests a selective, centrally mediated antitremor effect of HA-966.

The mechanism by which chemical agents can induce tremors is poorly understood. Since it has been shown by Chalmers & Yam (1962) that tremors below the level of spinal section can be induced by tremorine, it is doubtful whether the tremorine or oxotremorine-induced tremors are caused by an influence on basal ganglia which are the diseased sites of clinical Parkinsonism. It is conceivable that each type of chemically induced tremor represents a separate pharmacological model for different types of extrapyramidal disorders and this may form the basis for explaining the different potency of drugs used against Parkinsonism and other extrapyramidal conditions. Knowledge of the precise mechanism of tremor inhibition by

drugs is also scanty. While it has been stated that there is a good correlation between the anticholinergic and antitremor effect of atropine-like compounds (Ahmed & Marshall, 1962), other authors were unable to find such a correlation (Farquharson & Johnston, 1959). Even more puzzling is why such drugs provide symptomatic relief of clinical extrapyramidal conditions, in which so far no biochemical evidence for a primary hyperactivity of a central cholinergic mechanism has been found.

It has, however, been shown that there is a close correlation between abnormalities of the dopamine system and clinical conditions of extrapyramidal origin. Low concentrations of striatal and urinary dopamine were found in hypokinetic extrapyramidal conditions, while excess of dopamine and/or overstimulation of dopamine receptors may occur in hyperkinetic extrapyramidal syndromes (Barbeau, Murphy & Sourkes, 1961 ; Hornykiewicz, 1966 ; Calne & Sandler, 1970). In view of this correlation it was particularly interesting to observe that a potent tremor-inhibiting drug such as HA-966, selectively increased the striatal dopamine content, while not affecting noradrenaline or 5-hydroxytryptamine. In experiments published elsewhere (Hillen & Noach, 1971a) it has been shown that HA-966 does not activate enzymes concerned with dopamine biosynthesis, nor does it inhibit enzymes responsible for its breakdown. It could, however, be demonstrated (Hillen & Noach, 1971b) that after HA-966 administration the content of homovanillic acid, the major extraneuronal metabolite of dopamine, decreases in the corpus striatum, while the intraneuronal metabolite dihydroxyphenylacetic acid increases. Since no inhibition of COMT was found, all these data together indicate that either release of dopamine from striatal neurones is inhibited, or its re-uptake is enhanced. The later possibility seems to be less likely.

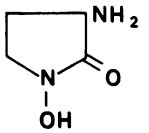
The fact that a compound, which has obvious antitremor activities both in animals and man, also inhibits net dopamine release from striatal neurones indicates that the latter mechanism may be of benefit to extrapyramidal patients with hyperkinetic rather than hypokinetic symptoms.

A further interesting finding with HA-966 was its pronounced muscle relaxing property. Since the compound failed to inhibit muscle activity when central connexion of the motor nerve was dissected, there is clear evidence that its relaxant effect is not due to a block of neuromuscular transmission, but originates from a central mechanism. The marked inhibition of the polysynaptic flexor reflex by HA-966 strongly suggests an effect on the internuncial neurones. It is thus possible that the depressing effect of HA-966 on the interneurones is a contributory factor in its antitremor activity as well as in its inhibitory effect on sensory arousal.

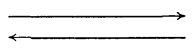
It was a consistent observation during experiments with HA-966, that there was a delay in the onset of action following intravenous administration. This observation suggested that a metabolic conversion of the drug was essential for a number of its effects. Experiments showing reduced activity of the compound in hepatectomized mice confirmed this idea and indicated the liver to be the site of metabolic conversion. Although we have not yet succeeded in identifying such a metabolite, it is tempting to explain the delay of onset on the basis of the chemical analogy between HA-966 on the one hand and the cyclic anhydric form of GABA and HOBA on the other hand. This analogy is shown as follows:

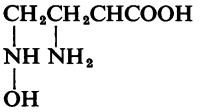
Cyclic anhydric form *Chain form*

(i)



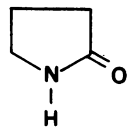
1-Hydroxy-3-amino-pyrrolidone-2
(HA-966)



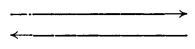


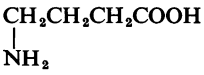
N-Hydroxy-α, γ-diaminobutyric acid
(hypothetical compound)

(ii)



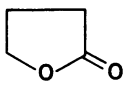
Pyrrolidone-2



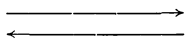


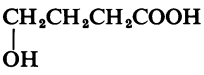
γ-Aminobutyric acid (GABA)

(iii)



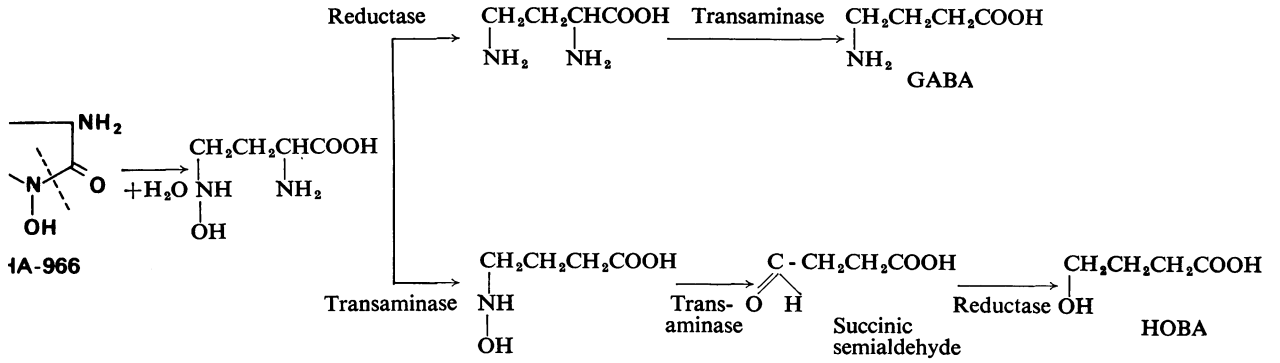
Butyrolactone





γ-Hydroxybutyric acid (HOBA)

Certain pharmacological actions of high doses of GABA and HOBA are similar to those observed with HA-966. Centrally mediated muscle relaxation by GABA and HOBA has been shown by Basil, Blair & Holmes (1964), and the role of HOBA as a metabolite involved in CNS depression states has repeatedly been proposed since the work of Bessman & Fishbein (1963). It has also been shown that HOBA can produce a sleep-like state simultaneously with a remarkably persistent righting-reflex (Basil *et al.*, 1964), which is strikingly similar to the behavioural effects of HA-966. More recently GABA, HOBA and some other structurally related analogues were shown to increase selectively striatal dopamine levels (Gessa, Spano, Vargiu, Crabai, Tagliamonte & Mameli, 1968). Inhibition of dopamine release from striatal neurones has also been suggested for butyrolactone (Roth & Suhr, 1970). A similar mechanism is implicated by us for HA-966. Impaired passage of GABA and HOBA through the blood-brain barrier explains why very high doses were required to obtain effects similar to those of HA-966. It is thus conceivable that metabolic conversion of HA-966 yields a material, which due to its structural analogy and easy penetration through a normally functioning blood-brain barrier, induces effects resembling those of GABA and HOBA. Two alternative pathways for such a metabolic conversion could be as follows:



Experimental work to prove or disprove the hypothetical conversion of HA-966 is presently in progress in our group.

The authors are grateful to Prof. Dr. E. Havinga, Dr. K. E. Kerling and to Dr. S. J. Roorda (Organic Chemistry Department, University of Leiden), who synthesized the first batch of HA-966, and to Mr. W. J. van der Burg (Organon, Oss) who supplied further amounts of the compound. The clinical studies mentioned in this paper were organized and the data collected by Dr. K. H. M. van der Velden (Organon, Oss). The experimental work in this study was completed while I.L.B. was affiliated to N.V. Organon (Oss). The dopamine experiments form part of a thesis by F.C.H.

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