Br. J. Pharmac. (1982), **76**, 439–445

EFFECTS OF 4-AMINOPYRIDINE ON ACETYLCHOLINE OUTPUT FROM THE CEREBRAL CORTEX OF THE RAT *in vivo*

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1 The effects of 4-aminopyridine (4AP) on the output of acetylcholine (ACh) from the cerebral cortex were investigated in unanaesthetized freely moving rats and in anaesthetized rats by means of the 'cup technique'. ACh was determined by bioassay on the dorsal muscle of the leech.

2 In unanaesthetized rats intraperitoneal injection of 4AP (3 mg/kg) had no effect on the cortical output of ACh.

3 After injection of morphine (10 mg/kg s.c.), which depressed the spontaneous output of ACh, 4AP increased the cortical output to a level significantly higher than that determined before morphine injection.

4 In rats anaesthetized with either urethane or pentobarbitone, drugs known to decrease cortical output of ACh, 4AP (i.v. or i.p.) elicited a significant increase in the output of ACh. The time-courses of the 4AP-induced effects were different depending on the anaesthetic drug used: an immediate increase slowly fading in urethane anaesthesia and a gradual increase after delayed onset in pentobarbitone-anaesthetized rats.

5 In some urethane-anaesthetized rats, respiratory frequency was kept constant (tracheotomy, connection to respirator, bilateral vagotomy) and prazosin (1 mg/kg i.v.) was administered to reduce the 4AP-induced increase of blood pressure. Cortical output of ACh was not related to changes in blood pressure. Moreover, the 4AP-induced increase in cortical ACh output was not related to changes in respiratory frequency.

6 In summary systemic administration of 4AP in subconvulsive doses (1 and 3 mg/kg) increased cortical output of ACh in rats anaesthetized with urethane or pentobarbitone or after injection of morphine, but not in untreated freely moving rats. It is suggested that the anaesthetic agents and morphine may cause an imbalance between excitatory and inhibitory central pathways, and that this imbalance may play a role in their depressant effect on cortical output of ACh and/or in the 4AP-induced facilitation described in this paper.

Introduction

Aminopyridines facilitate transmitter release by promoting voltage-dependent calcium influx (see Thesleff, 1980; Löffelholz & Weide, 1982). It is assumed that this effect is secondary to a blockade of the voltage- and time-dependent outward potassium current ('delayed rectification' of action potential) of the nerve terminals. Direct measurements of the facilitation by aminopyridines of acetylcholine (ACh) output evoked by electrical nerve stimulation have been carried out on the guinea-pig ileum (Vizi, Van Dijk & Foldes, 1977), on the perfused chicken heart (Weide & Löffelholz, 1980) and on the rat diaphragm (Gundersen & Jenden, 1981).

It has been found that intravenous injection of 4-aminopyridine (4AP, 1 mg/kg) markedly increases mono- and polysynaptic spinal reflex discharges and enhances transmission in both excitatory and inhibitory synapses, whereas the passive electrical properties of the soma-dentritic membrane and receptor sensitivity of motoneurones remains unchanged (Jankowska, Lundberg, Rudomin & Sykova, 1977). In high doses, 4AP causes convulsions probably due to effects on both cortical (Baranyi & Fehér, 1979) and spinal neurones.

The penetration of 4AP from the blood into the central nervous system seems to be rapid. It has been shown that 5 min after an intravenous injection of 7 mg/kg 4AP into rats anaesthetized with urethane, the concentration of the drug in the cerebrospinal fluid reached about one-fifth of that of the serum (Lemeignan, Millart, Letteron, Lamiable, Josso, Choisy & Lechat, 1982).

0007-1188/82/070439-07 $01.00

Reviewing the rapidly growing literature on the neurophysiological and clinical effects of aminopyridines, it is striking that pronounced effects on peripheral and central neurones have been observed frequently in situations of apparant synaptic depression (see Discussion). For example, the drug was introduced into anaesthetics as an analeptic agent to shorten the recovery time after ketamine-diazepam (Agoston, Salt, Erdmann, Hilkemeijer, Bencini & Langrehr, 1980) or barbiturate anaesthesia (Rao, Nagashima, Deery & Foldes, 1977) and to reverse fentanyl-induced respiratory depression (Sia & Zandstra, 1981).

In the present work, the effect of 4AP on output of ACh from the cerebral cortex *in vivo* was studied in rats by direct determination of ACh. The animals were untreated or treated with morphine, urethane or pentobarbitone.

Methods

Adult male Wistar rats weighing 250–350 g were used.

Unanaesthetized rats

Under ketamine anaesthesia (100 mg/kg i.p.) a Perspex cylinder was screwed through the left or right parietal bone so as to exert a slight pressure on the *dura mater* as described by Casamenti, Pedata, Corradetti & Pepeu (1980). The output of ACh was investigated 3 days after surgery. During this time, the *dura* was repeatedly washed by a terramycin solution (300 μg/ml) up to 2 h before the experiment.

Anaesthetized rats

Rats were anaesthetized either with urethane (1.25 g/kg i.p.) or with pentobarbitone (initially 45 mg/kg i.p. and later 6 mg/kg i.m. after periods of about 30 min). The output of ACh was investigated by the cortical cup technique (Mulas, Mulas & Pepeu, 1974). The skull was opened and a small (0.25 cm^2) Perspex cylinder containing Ringer solution was placed on the exposed cortex after removing the *dura mater*. The body temperature was maintained at 37°C by a heating pad with a rectal probe.

Sampling procedure and acetylcholine estimation

The Ringer solution placed in the cylinder had the following composition (mmol/l): NaCl 150, KCl 5.6, $CaCl_2$ 1.6, $NaHCO_3$ 5.9, glucose 5.5 and physostigmine 0.30. The volume of the solution used was 0.3 ml both in the unanaesthetized and in the anaesthetized rats. Every 10 min the solution was removed from the cylinder, diluted as appropriate and bioassayed for acetylcholine on the dorsal muscle of the leech (*Hirudo medicinalis*) according to the method of Murnaghan (1958). All results are expressed in terms of acetylcholine chloride.

Respiratory rate control and blood pressure monitoring

Bilateral vagotomy at the level of the cervical vagus nerves was carried out 30 min before collection of the samples in order to block peripheral reflex mechanisms. In these experiments, rats had been tracheotomized and were connected to a respirator (80–84 breaths/min)to ensure constant controlled respiration.

Mean arterial blood pressure was measured by means of a pressure transducer from the right carotid artery and monitored on a pen recorder.

Drugs

The drugs used and their sources were: urethane (Merck), pentobarbitone (Abbott), ketamine (Parke Davis), 4-aminopyridine (Sigma), prazosin (Boehringer Ingelheim) and morphine chloride (Carlo Erba). Drugs were injected intravenously, intraperitoneally or subcutaneously, dissolved in 0.9% w/v NaCl solution (saline) in a volume of 0.3 ml or less, except urethane which was dissolved in about 2 ml.

Statistics

Mean values are presented with their standard errors and Student's *t* test (paired or unpaired) was used for comparison. *P* values of less than 0.05 were considered significant.

Results

Unanaesthetized rats

In 4 unaesthetized rats (with the cylinder installed), 4AP (3 mg/kg i.p.) caused hypersalivation, chromodacryorrhea, tremors and muscle twitches; convulsions were absent. The cortical output of ACh (around 1.4 ng $min^{-1}cm^{-2}$) failed to increase upon injection of 4AP (see Figure 1).

Anaesthetized rats

It was found (Figures 2 and 3, Table 1) that 4AP increased the cortical output of ACh in rats anaesthetized with urethane or pentobarbitone. To rule out the possibility that increases in cortical output of ACh by 4AP were caused by changes in respiratory

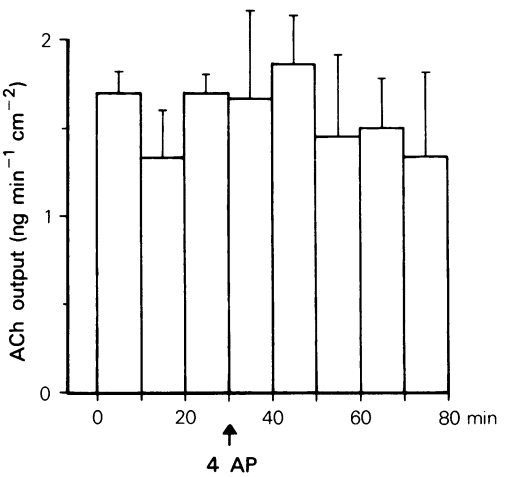

Figure 1 Effect of 4-aminopyridine (4AP) (3 mg/kg. i.p.) on the cortical output of acetylcholine (ACh) in unanaesthetized rats. 4AP had no significant effect on acetylcholine output (means of 4 experiments); vertical lines show s.e.mean.

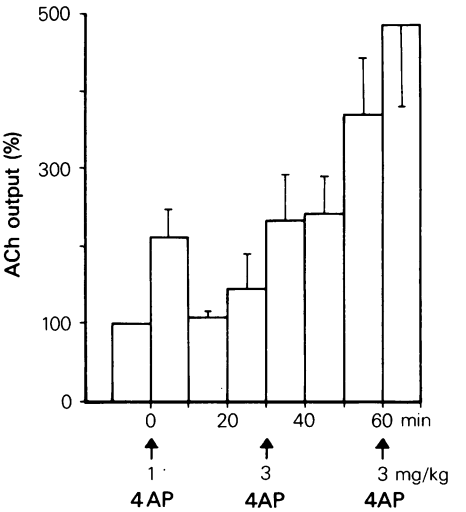

Figure 2 Effect of 4-aminopyridine (4AP) on cortical output of acetylcholine (ACh) in rats anaesthetized with urethane and subjected to 'treatment' (bilateral vagotomy, respiratory support, 1 mg/kg prazosin). Treatment was given 30 min before zero time. 4AP was injected intravenously at various times in doses as indicated in the graph (further details in Table 1). The ACh output was expressed as a percentage of the preinjection level (100%) obtained in each experiment as the mean of three consecutive samples immediately before the first injection of 4AP (means of 4 experiments are shown; vertical lines indicate s.e.mean).

rate and blood pressure, some of the animals (Figure 2, Table 1) were tracheotomized and, after vagotomy, ventilated with air by means of a respirator at a rate of 80–84 breaths/min. In addition to respiratory support they received an α-adrenoceptor blocking agent (prazosin, 1 mg/kg i.v.) to reduce the peripheral blood pressure effects (Bowman, Marshall, Rodger & Savage, 1981). In another group of animals (Figure 3, Table 1) no attempts were made to alter 4AP-induced effects on respiration and blood pressure. In the following description the animals of the two groups will be referred to as treated or untreated rats, respectively.

Figure 2 shows that in treated rats anaesthetized with urethane the injection of 1 mg/kg i.v. of 4AP induced an immediate and short lasting increase of ACh output from the cerebral cortex. The preinjection output of ACh (0.89 ± 0.15 ng min^{-1} cm^{-2}) has been observed over a period of 30 min during which it maintained an unchanged level (cf. also the basal output of ACh shown in Figure 3).

Table 1 Effect of 4-aminopyridine (4AP) on cortical acetylcholine (ACh) output and mean arterial blood pressure in rats anaesthetized with urethane

Groups of rats	*4AP* (mg/kg)	*ACh output* (ng min^{-1}cm^{-2})	*Blood pressure (mmHg)*[a] *Absolute*	*Δ max*[b]
I. Untreated	0	0.98 ± 0.33	103 ± 5	—
	3	2.21 ± 0.33*	131 ± 15*	37 ± 4*
II. Treated[c]	0	0.89 ± 0.15	59 ± 3†	—
(prazosin,	1	1.92 ± 0.29*	62 ± 4†	2 ± 1
vagotomy,	3	2.88 ± 0.36*	71 ± 8*†	13 ± 3*
respirator)	3	3.12 ± 0.87*	71 ± 6*†	19 ± 3*

[a]Mean arterial blood pressure (number of experiments $n = 3-5$) and ACh output ($n = 4-6$) measured in different animals. [b]4AP induced increase above preinjection level. [c]30 min before 4AP injection, animals were bilaterally vagotomized, ventilated (80–84 breaths/min; air) and received prazosin 1 mg/kg i.v. 4AP was injected in successive doses of 1,3 and 3 mg/kg i.v. every 30 min (see Figure 2). Given are means ± s.e.

*Significantly different from preinjection level of the same group ($P < 0.05$).

†Significantly different from group I preinjection level ($P < 0.05$).

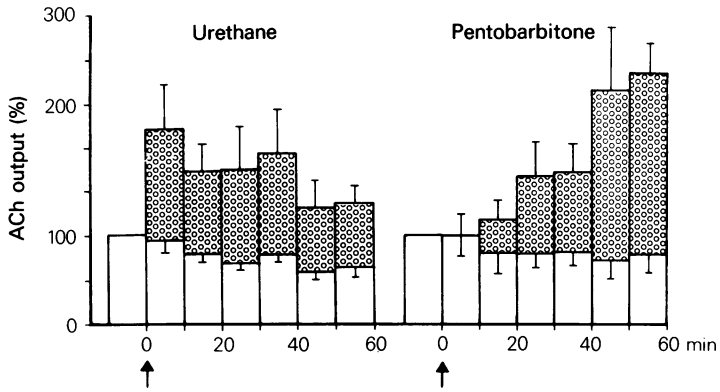

Figure 3 4-aminopyridine (4AP)-evoked increase of cortical output of acetylcholine (ACh) in rats anaesthetized with urethane or pentobarbitone. 4AP (3 mg/kg) or saline (basal ACh output) were injected intraperitoneally at zero time. Open columns: basal ACh output. Shaded columns:4AP-evoked increase. The ACh output was expressed as a percentage of the preinjection level (further details in Figure 2). (Means of 5–9 experiments; vertical lines show s.e.mean).

Figure 2 also shows that a subsequent injection of 4AP (3 mg/kg i.v.) elicited a further immediate increase of cortical ACh output which did not fade within the period of observation (30 min). A further adminstration of 4AP (3 mg/kg i.v.) again raised the output which now amounted to nearly 500% of the preinjection level (Figure 2). The time-course after the third injection was not followed beyond 10 min (1 collection sample) because the animals exhibited marked muscular twitches; voluntary movements were not observed.

The discrepancy between the findings in unaesthetized and anaesthetized animals led us to investigate whether the effect of 4AP observed in the presence of urethane was due specifically to urethane or was a more general effect of anaesthetics. For this purpose we compared the effects of 4AP (3 mg/kg i.p.) in urethane- and pentobarbitone-anaesthetized rats.

At first, the basal output of ACh from the cerebral cortex of the untreated rats anaesthetized with either urethane or pentobarbitone was studied following the injection of drug-free saline(i.p.). As shown in Figure 3, the basal output remained fairly stable over the period of observation (90 min); there was a small decline between 20 and 60 min.

In urethane-anaesthetized rats, administration of 4AP (3 mg/kg i.p.) elicited a significant increase in the output of ACh within 10 min; this was followed by a slow return towards the preinjection level. In the individual animals, the peak effects occurred between zero and 40 min after administration of 4AP and reached an average value of $244 \pm 35\%$ ($n = 5$) of the preinjection level (100% equivalent to 0.98 ± 0.16 ng $min^{-1} cm^{-2}$).

In rats anaesthetized with pentobarbitone, 4AP (3 mg/kg i.p.) increased cortical ACh output, but the time-course was different from that seen under urethane anaesthesia. The output of ACh increased gradually during 1 h. This effect was not followed beyond 1 h because of difficulties in maintaining a constant anaesthesia with pentobarbitone in these experiments. In these rats, the peak increase occurred between 40 and 60 min after 4AP injection and reached an average level of $392 \pm 71\%$ ($n = 6$) of the preinjection output (100% equivalent to 1.83 ± 0.33 ng min^{-1} cm^{-2}). The time-course of the 4AP-induced increase in cortical output of ACh did not depend on the route of administration (i.v. or i.p. injections; data not shown).

In rats anaesthetized with either urethane or pentobarbitone injection of 4AP led to instantaneous micturition, diarrhoea,hypersalivation, increase in respiratory rate and muscle twitches.

4-Aminopyridine-induced blood pressure changes in comparison with cortical acetylcholine output

The mean arterial blood pressure in the right carotid artery was monitored before and after administration of 4AP in urethane-anaesthetized rats that had been treated (prazosin, vagotomy, respirator) or remained untreated. As shown in Table 1, the basal blood pressure was lowered by the treatment from 103 ± 5 mmHg to 59 ± 3 mmHg ($n = 5$). This decrease in blood pressure was not accompanied by a change in the cortical output of ACh. Moreover, the administration of successive doses of 4AP caused only slight increases in the blood pressure when compared to the effect in untreated rats. In contrast the effects of 4AP (1, 3 and 3 mg/kg i.v.) on cortical ACh output seemed more pronounced in treated

than in untreated rats. Moveover, in treated rats 4AP (1 mg/kg i.v.) failed to increase blood pressure but doubled cortical output of acetylcholine. Thus, in our experiments, changes in cortical output of acetylcholine were not related to changes in blood pressure.

Unanaesthetized rats after injection of morphine chloride

Since morphine, like urethane and pentobarbitone, decreases cortical ACh output (see Discussion), we examined whether an acute injection of morphine chloride into freely moving rats has a similar effect to urethane and pentobarbitone in facilitating 4AP-induced ACh output from the cerebral cortex. The results of this experiment are shown in Figure 4.

When the spontaneous output of ACh (around 1.7 ng $min^{-1}cm^{-2}$) was significantly ($P < 0.05$) decreased by administration of morphine (10 mg/kg s.c.), 4AP (3 mg/kg i.p.) not only reversed the morphine-induced depression but increased the ACh output to a level considerably above the basal output recorded before administration of morphine ($P < 0.05$).

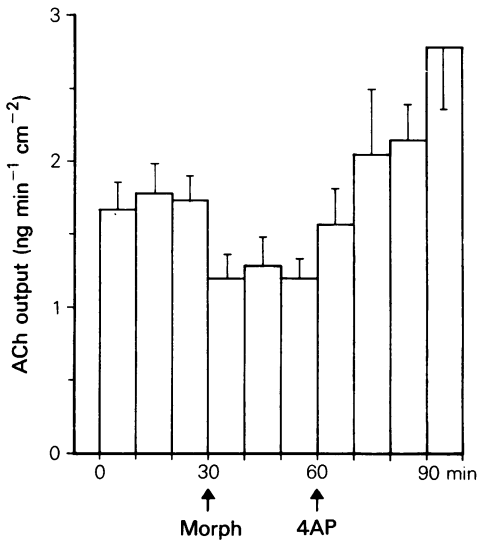

Figure 4 4-Aminopyridine (4AP)-evoked increase of cortical output of acetylcholine (ACh) in unanaesthetized rats after morphine chloride (Morph) (10 mg/kg s.c.). 4AP (3 mg/kg) was injected intraperitoneally 30 min after morphine. Further details in Figure 1. (Means of 4 experiments; vertical lines show s.e.mean.)

Discussion

Our experiments show that in the rat, subconvulsive doses of 4AP (1 or 3 mg/kg) enhanced output of ACh from the cerebral cortex *in vivo*. However, this effect was only apparent in rats anaesthetized with urethane or pentobarbitone or after injection of morphine chloride, i.e. after a treatment which is known to depress cortical ACh output. General anaesthesia reduces the output of ACh from the cortex of cats and sheep (Mitchell, 1960), and morphine (1 to 15 mg/kg, i.p.) markedly decreases the ACh output from the cerebral cortex of the guinea-pig (Beani, Siniscalchi & Sarto, 1979) and rat (Casamenti *et al.*, 1980). It has been shown that pentobarbitone, urethane and morphine increase the ACh content in the brain, presumably as a consequence of a reduced release of ACh (Giarman & Pepeu, 1962; Pepeu, 1965). The increase was found to be due to an accumulation of ACh in the nerve endings (Beani, Bianchi, Megazzini, Ballotti & Bernardi, 1969).

In the present experiments, there was no obvious depressant effect of the anaesthetics on ACh output from the cerebral cortex (output in unanaesthetized rats: 1.5 ng $min^{-1}cm^{-2}$; output in urethane-anaesthetized rats: 1.0 ng $min^{-1}cm^{-2}$; output in pentobarbitone-anaesthetized rats: 1.8 ng min^{-1} cm^{-2}). One has, however, to take into consideration that overflow of ACh released from cortical neurones into the solution in the cup is reduced by the *dura mater* (Beani, Bianchi, Santoniceto & Marchetti, 1968), which was removed in anaesthetized rats. Thus the recovery of released ACh in the solution must be higher in anaesthetized than in unanaesthetized rats.

The present data suggest that the increase by 4AP of the cortical output of ACh in rats may be dependent on a depressed rate of ACh release and possibly also on the mechanism which produces the depression of release. This hypothesis is derived, firstly, from the lack of an increase in ACh release in response to 4AP in unanaesthetized rats and, secondly, from the different time-courses of the 4AP-evoked facilitation of ACh release observed in urethane- and pentobarbitone-anaesthetized animals. It is well known that the central events during general anaesthesia vary considerably according to the type of the anaesthetic drug and to the level of anaesthesia. It was, therefore, not surprising that the reversal by 4AP of the urethane and pentobarbitone effects on cortical output of ACh exhibited marked differences in their time-courses (Figure 3): the facilitation was immediate, rapidly reached a maximum and gradually declined in urethane anaesthesia, whereas the effect was delayed and gradually increased in pentobarbitone anaesthesia. The rapid onset of the effect of 4AP in urethane experiments indicates a rapid diffusion of 4AP into the central nervous system,

which is in agreement with recent pharmacokinetic data (see Introduction).

However, it could be argued that facilitation of cortical output of ACh by 4AP was caused indirectly by effects on blood pressure and respiration. Experiments designed to rule out this possibility were carried out in urethane anaesthetized rats after bilateral vagotomy, institution of artificial respiration and injection of the α-adrenoceptor blocking drug, prazosin. (Phentolamine, another α-blocking agent, had been shown to block the increase in arterial blood pressure produced by 4AP in the dog; see Bowman *et al.*, 1981). Hence, the treatment blocked vagus-mediated reflexes, kept frequency of respiration constant and caused a marked reduction of 4AP-induced effects on blood pressure. This experiment showed that changes in cortical output of ACh were not related to changes in blood pressure under our conditions (Table 1). The finding corroborates earlier observations in the rat (Hemsworth & Neal, 1968) and in the cat (Pepeu & Bartolini, 1968). Therefore we assume that the 4AP-induced increases in cortical ACh output in anaesthetized rats are caused at a central site of action.

After we had detected the modifying effect of the two general anaesthetics on the 4AP-induced facilitation of cortical output of ACh, it was interesting to study whether morphine also modifies the effect in unanaesthetized rats. Although morphine is not a general anaesthetic drug, it decreases, like urethane and pentobarbitone, the cortical output of ACh in unanaesthetized (Figure 4; Casamenti *et al.*, 1980) and in anaesthetized rats (Jhamandas & Sutak, 1974; Pepeu, Garau, Mulas & Marconcini-Pepeu, 1975). In the present experiments 4AP increased the cortical output of ACh after injection of morphine to a rate significantly above the preinjection level (Figure 4). According to the protocols of our previous experiments using identical conditions (Casamenti *et al.*, 1980), the depressant effect of morphine on cortical output of ACh faded gradually. After complete recovery, the output did not exceed the preinjection level until the end of the experiment (80 min after morphine injection) indicating the absence of a rebound increase in the cortical ACh output following depression of release. It is concluded that morphine injection had a facilitatory effect on the 4AP-evoked increase in the cortical output of ACh.

Of course, we cannot localize the central site of 4AP action leading to facilitation of the cortical ACh output, since the cortical cholinergic network is activated or inactivated by neuronal pathways impinging upon cholinergic neurones. Although a direct antagonism between pentobarbitone and 4AP on the evoked release of ACh has been described for the postganglionic parasympathetic neurone of the heart (Weide & Löffelholz, 1980), the net effect of 4AP on cortical ACh output produced by its interactions with urethane, pentobarbitone and morphine may reflect complex central actions.

It has been suggested that these central net effects of 4AP on transmitter release may be useful in the search for neuronal interactions (Löffelholz & Weide, 1982). Thus administration of 4AP may raise the activity of central excitatory and inhibitory neurones in parallel to a higher level, leaving their functional integrity intact (Jankowska *et al.*, 1977). Interference with one of the two systems could play an essential role in the modifying effect of general anaesthetics and morphine on cortical output of ACh induced by 4AP.

This research was supported by grant No. CT 80.003 94.04 from C.N.R. and by grants from the Deutsche Forschungsgemeinschaft (Lo 196). R.C. is recipient of an Alexander von Humboldt-Stiftung research fellowship. We thank Boehringer-Ingelheim for kindly donating prazosin.

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(Received December 15, 1981.
Revised February 15, 1982.)