10^{-4} M picrotoxin, bicuculline or one of the bicylic phosphorous esters 4-ethyl-1 phospha 2,6,7-trioxabicyclo (2,2,2) octane (EPTBO) or its isopropyl equivalent (IPTBO).

Evoked presynaptic inhibition was estimated quantitatively as the area enclosed by the large positive-going component (P wave) of the cuneate evoked potential, which is known to reflect the underlying depolarization of primary afferent terminals, the basic mechanism of vertebrate presynaptic inhibition. Alterations in the size of the P wave have frequently been used in pharmacological investigations of presynaptic inhibition[11] but computer-averaging of the evoked response adds greater reliability to the technique.

Results and discussion. Both EPTBO and IPTBO were effective antagonists of presynaptic inhibition, reducing or abolishing the cuneate P wave. In both cases the onset of action was apparent within a few min of the superfusion fluid being applied to the cuneate and the maximum effect was reached within 30–40 min for 10^{-3} M EPTBO (figure) and 10^{-4} IPTBO, the latter being consistently about 10 times more potent than EPTBO. After removal of EPTBO or IPTBO from the superfusion fluid, the depressant effects were fully reversible within a period of about 2 h. There was no consistent effect on the preceding N wave component of the evoked response (figure). The depressant action of picrotoxin on the P wave was approximately equipotent with that of EPTBO, bicuculline with IPTBO. In contrast to the bicyclic phosphorous esters, however, the actions of picrotoxin and bicuculline were either only partially reversible or irreversible over the time course of the experiments, as has been reported elsewhere[5,6].

The bicyclic phosphorous esters are potent convulsants, block the depressant actions of GABA on single neurones in the rat medulla and the depolarizing action of GABA on superior cervical ganglion cells with the relative potencies of IPTBO = 1, EPTBO = 0.1[9]. The data reported here extend these observations to include antagonism of presynaptic inhibition with the relative potencies remaining unchanged. It is likely that the convulsant activity of the bicyclic phosphorous esters depends to a considerable extent on their ability to antagonize presynaptic inhibition. The observations reported here also support the theory that GABA is the presynaptic inhibitory transmitter[6].

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The effect of ion pair formation on the antimuscarinic activity of methantheline

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Summary. The quaternary ammonium compound, methantheline, was found to antagonize acetylcholine induced contractions in isolated guinea pig ileum by a mechanism which did not conform to the criteria for either competitive or non-competitive inhibition. Enhancement of the lipid solubility of methantheline by formation of an ion pair with trichloracetate failed to influence its cholinergic inhibitory activity. The results suggest that in the guinea pig ileum a) an intracellular site of action does not exist for methantheline and b) the membrane receptors for methantheline most likely are located in an aqueous environment.

Ion pairing is a technique which has been used to mask the charged site on an ionic species and thereby render it more lipid soluble[3–5]. The potential application of this technique as a means of improving the gastrointestinal absorption of highly ionized drugs has been demonstrated by Irwin et al.[6] who observed an increase in rate of onset and in intensity of mydriatic response following oral administration of the quaternary ammonium compound isopropamide iodide, in combination with the ion pair forming counter ion trichloracetate. A change in the physical properties of a charged drug molecule conceivably could influence its pharmacologic activity in other ways such as by increasing its accessibility to intracellular sites of action or by altering its affinity for membrane receptors. The present investigation was undertaken to evaluate the effects of ion-pair formation on the lipid solubility and antimuscarinic activity of methantheline bromide, a quaternary ammonium compound which is highly ionized at physiological pH.

Materials and methods. The effect of ion pair formation on lipid solubility was examined by 2 methods. As a direct measurement, the partition coefficient for methantheline was determined in the presence of excessive molar concentrations of trichloroacetate (TCA). An indirect assessment of the ability of complex formation with TCA to enhance the lipid solubility of methanteline was made using an in situ rat intestinal loop preparation[7]. 4.76 mM ^{14}C-methantheline (specific activity, 1.25×10^7 cpm/mg) in the presence of excessive molar concentrations of TCA was introduced into the intestinal lumen and the amount remaining was determined at different times over a 1-h-period using a Packard Tricarb liquid scintillation counter.

The effect of ion pair formation on the antimuscarinic activity of methantheline was investigated by the following procedure. Female guinea-pigs weighing 200–300 g were sacrificed by cervical dislocation and the abdominal cavity opened by midline laparotomy. A 2–3 cm segment of distal ileum was excised and trimmed of attached mesentery. The sample was suspended in a 15 ml isolated tissue bath containing Tyrode's solution of the following millimolar composition: NaCl, 137; KCl, 2.7; $CaCl_2$, 2.5; $NaHPO_4$, 4.2; $MgSO_4$, $7H_2O$, 8.3; glucose, 10; $NaHCO_3$, 14. The bath was maintained at 37 ± 1 °C and continuously bubbled with 95% O_2–5% CO_2. The system was arranged in such a way that washout of drug solutions could be accomplished without removing tissue samples from the bath. Contractions induced by varying doses of acetylcholine chloride were monitored using a Grass FT03C force-tension transducer in conjunction with a Hewlett-Packard 7404A oscillographic recorder.

An ion pair solution was prepared by combining meth-

antheline bromide with a 25 molar excess of TCA neutralized with sodium hydroxide. Sodium chloride was withdrawn from the Tyrode's solution to compensate for the osmotic change caused by the addition of the methantheline-TCA ion pair. After a series of contractile responses to varying doses of acetylcholine had been recorded, the tissue was exposed to Tyrode's solution containing either methantheline or methantheline-TCA. In all experiments, the bath concentration of methantheline was 10 µg/ml. 5 min thereafter, a second series of contractile responses to acetylcholine were obtained.

Results and discussion. The data shown in tables 1 and 2 clearly demonstrate that ion pair formation with TCA enhances the lipid solubility of methantheline. The partition coefficient of the drug was observed to increase with increasing excessive molar concentrations of TCA (table 1). Likewise, the amount of methantheline remaining in the in situ intestinal loop was observed to decrease with time as a function of TCA concentration (table 2).

Both methantheline and methantheline-TCA were effective in inhibiting acetylcholine induced contractions. These effects were completely reversible in both cases as evidenced by a return to 100% of pre-drug response after 3–4 min washing with drug-free Tyrode's solution. Figure 1 illustrates the regression lines calculated from log dose-response data for acetylcholine alone and for acetylcholine in the presence of methantheline or methantheline-TCA. The ED_{50} for acetylcholine alone was 2.8×10^{-5} mg/ml. The presence of methantheline or methantheline-TCA caused a shift to the right in the acetylcholine log dose-response curve. The corresponding ED_{50} values were 1.4×10^{-1} and 3.6×10^{-1} mg/ml respectively. There was no statistically significant difference ($p > 0.05$) between the ED_{50} values for acetylcholine in the presence of the 2 forms of the drug.

A statistical test for parallelism revealed that the regression lines for acetylcholine in the presence of methantheline and methantheline-TCA were parallel. However, when the regression line for acetylcholine alone was compared to that for acetylcholine in the presence of either methantheline or methantheline-TCA, the slopes were found to differ significantly. This lack of parallelism indicates that the agonist-antagonist interaction did not involve a direct competitive mechanism. Further, criteria for non-competitive inhibition were not fulfilled since high doses of acetylcholine effectively overcame the inhibition exerted by methantheline or methantheline-TCA. Similar anomalous behavior has been described for other quaternary ammonium compounds and the concept of allosteric regulation has been advanced by Freeman and Turner to explain the phenomenon[8]. According to their hypothesis, quaternary ammonium compounds bind to an anionic site in the vicinity of the cholinergic receptor and from this position exert a regulatory effect on the receptor.

Ion pair formation increases the partitioning of a charged species into a nonaqueous phase by effectively masking lipophobic sites and thereby increasing lipid solubility[9]. It may be assumed that in the present experiments a significant amount of methantheline would enter the intracellular space in the presence of the ion pair forming

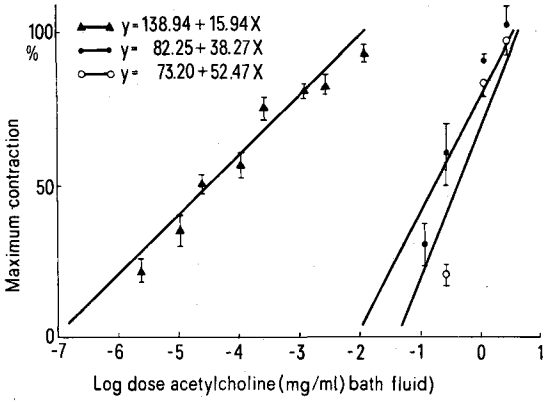

Fig. 1. The response of the isolated guinea-pig ileum to acetylcholine alone (▲—▲) and to acetylcholine in the presence of methantheline (●—●) or methantheline-TCA (○—○). Each point represents the mean ± SEM from at least 6 experiments.

Table 1. Partition coefficients for methantheline bromide alone and in the presence of various concentrations of TCA

Excess TCA (Mol. conc.)	Partition coefficients
–	0.03
5 Mol	1.58
10 Mol	3.10
25 Mol	6.72

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Table 2. mM conc. of methantheline remaining in the intestinal loop in the presence of various concentrations of trichloracetate

	Time (min)								
Solution	0	5	10	15	20	30	40	50	60
10fold Molar excess TCA	4.76	4.51	4.13	4.18	3.99	3.96	3.64	3.52	3.42
		(0.13)	(0.20)	(0.23)	(0.23)	(0.26)	(0.23)	(0.23)	(0.24)
	4.76	2.66	1.77	1.20	0.86	0.56	042	0.33	0.29
25fold Molar excess TCA		(0.11)	(0.07)	(0.05)	(0.03)	(0.02)	(0.02)	(0.02)	(0.02)

n = 4; numbers in parentheses are ± SEM.

counter ion, TCA. If intracellular receptor site exists for methantheline, as is the case for the tertiary amine local anesthetics such as procaine[10,11] then a characteristic deviation in the log dose-response curve for acetylcholine should occur under conditions which improve the drug's accessibility to the intracellular space. However, as has been stated, the regression lines for acetylcholine in the presence of methantheline and methantheline-TCA did not differ statistically from one another. In light of the fact that the antimuscarinic activity of methantheline was unaffected by enhancing the drug's lipid solubility, it is unlikely that an intracellular locus of action exists. In addition, some inferences may be made concerning the nature of the membrane receptor environment for methantheline. Under the conditions employed in these experiments, the methantheline-TCA ion pair would exist only in a non-aqueous medium such as the cell membrane. Consequently, if the methantheline receptors were located in an aqueous environment, only free drug would be available and no further deviation in the log-dose response curve for acetylcholine would be expected when TCA was present[12,13]. The results presented in the figure support the hypothesis that formation of an ion pair with TCA did not influence the antimuscarinic activity of methantheline in the isolated tissue preparation.

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Inhibitory tripeptide, Lys-Phe-Tyr, as a fragment of physalaemin

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3 December 1976

Summary. Several peptides were separated from α-chymotrypsin-treated physalaemin by high voltage paper electrophoresis, and inhibition of the excitability of a molluscan giant neurone (tonically autoactive neurone) by the tripeptide, Lys-Phe-Tyr, was demonstrated.

Physalaemin is a hypotensive endecapeptide isolated by Erspamer et al.[2,3] from amphibian skin. Its biphasic effect (inhibitory-excitatory) on the excitability of a giant neurone (the TAN, tonically autoactive neurone)[4,5] in the subesophageal ganglia of the African giant snail (Achatina fulica Férussac) has been reported previously[6]. Excitation was much more marked than inhibition. Enzyme treatment of physalaemin showed that marked inhibition of the TAN could be produced by the α-chymotrypsin-(CT)-treated physalaemin[7]. In the present study, each fragment of this CT-treated physalaemin was separated by high voltage paper electrophoresis. Of the separated fragments, a tripeptide (Lys-Phe-Tyr) and an octopeptide including the tripeptide (Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr) (Pyr: L-pyroglutamic acid) were shown to have a strong inhibitory effect on the TAN.

Physalaemin (Protein Research Foundation, Osaka; 2.5×10^{-4} kg/l in 0.1 M ammonium acetate buffer, pH 8.2) was treated with CT (Worthington Biochemical Corporation, 55 units/mg; 5×10^{-5} kg/l; $^1/_{10}$ volume of physalaemin solution) at 37 °C for 6 h. After the treatment, trasylol (Bayer AG) was added to stop enzyme activity. High voltage paper electrophoresis of CT-treated physalaemin (Anastasi et al.[8]) was performed under the following conditions: paper, Toyo No. 51A; 50 V/cm; 90 min; pH, 1.9; running buffer, CH_3COOH–HCOOH(98%)–H_2O (87:25:888). The whole paper was stained with fluorescamine after electrophoresis, the guide strip being stained by ninhydrin and a peptide reagent[9]. Fragments of physalaemin were recovered from each of the peptide bands. The amino acid sequences of each fragment were determined after acid hydrolysis at 105 °C for 16 h.

A glass micropipette was implanted into the TAN soma, the intracellular biopotential recorded by a pen-writing galvanometer, and the number of spike discharges per minute counted with a spike counter. Substances to be examined were applied in 2 ways: by bath application (substances were dissolved in the snail's physiological solution[10], and applied to the dissected ganglia) and by microdrop application (a microdrop about 100 μm in diameter of the solution to be examined was formed at the tip of a second micropipette, and the microdrop placed precisely on the TAN surface) (diameter of this neurone was about 200 μm).

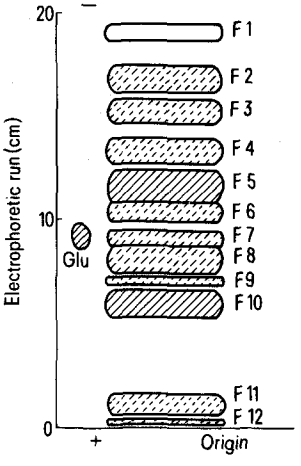

Fig. 1. Peptide bands of chymotrypsin-treated (for 6 h) physalaemin separated by high voltage paper electrophoresis (schematic drawing). Amino acid sequences of each fragment of physalaemin were determined to be as follows (Pyr: L-pyroglutamic acid): fragment (F) 1, Lys-Phe; F 2, Met-NH_2; F 3, Lys-Phe-Tyr; F 4, Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu; F 5, Gly-Leu-Met-NH_2; F 6, Gly-Leu-Met-NH_2; F 7, Tyr-Gly-Leu; F 8, Tyr-Gly-Leu-Met-NH_2; F 9, Pyr-Ala-Asp-Pro-Asn-Lys-Phe; F 10, Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr; F 11, Pyr-Ala-Asp-Pro-Asn and Gly-Leu-Met; F 12, Pyr-Ala-Asp-Pro-Asn and Gly-Leu-Met.