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EFFECT OF INHIBITORS OF CHOLINE ACETYLATION ON ACETYLCHOLINE OUTPUT AND MOTILITY IN RESPONSE TO ANTICHOLINESTERASES AND TO DISTENSION OF THE LUMEN OF ISOLATED GUINEA-PIG ILEUM

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Some pharmacological effects of acyl-aryl compounds, which inhibit the enzymatic acetylation of choline, have been studied on the isolated guinea-pig ileum. Diphenylylbutylacetic acid (M.G. 1763) greatly reduced the output of acetylcholine from intestine. The intestinal contractions produced by anticholinesterase drugs were inhibited, while the response to acetylcholine itself was little affected. The contraction of longitudinal muscle elicited by raising intra-luminal pressure was also decreased, while the emptying phase of the peristaltic reflex was scarcely affected. There was a correlation between inhibitory activity and chemical structure.

In previous work (Garattini, Morpurgo, and Passerini, 1958) some acyl-aryl compounds were shown to inhibit acetylcholine synthesis *in vitro* using choline acetylase extracted from rabbit brain, and evidence was advanced that this inhibition was effected by preventing acetylation of coenzyme A. In order to study the pharmacological actions of these compounds, experiments have been performed on the isolated intestine which is known to synthesize large amounts of acetylcholine (Feldberg and Lin, 1949).

The effect of these drugs has been studied both on the release of acetylcholine from isolated guinea-pig ileum in the presence of eserine, and also on the intestinal contractions produced by anticholinesterase drugs which have been attributed to the accumulation of acetylcholine in the wall of the intestine (Koelle, Koelle and Friedenwald, 1950; Shelley, 1955; Admiral, Myers and Van Houten, 1955). Other experiments were performed on the peristaltic reflex elicited by distension of the lumen in the isolated guinea-pig ileum since it has been shown that distension of the lumen provokes an increased output of acetylcholine (Chujyo, 1952) which is followed by a contraction of the longitudinal muscle.

METHODS

Assay of Acetylcholine Released from the Isolated Ileum of the Guinea-pig.—Guinea-pigs were killed by a blow on the neck and bled. The distal portion of ileum was rapidly removed and a segment about 15 cm. in length was washed, tied at both ends, and incubated in 20 ml. oxygenated, Mg-free Tyrode solution at 37°, and eserine sulphate was added (10 μg./ml.). Every 30 min. the bath fluid was exchanged and assayed for acetylcholine on the eserinized frog rectus muscle. At the end of the experiment, the intestine was taken out, blotted with filter paper, and weighed. The release of acetylcholine was expressed as μg./g. of tissue.

Responses of the Isolated Ileum of the Guinea-pig to Anticholinesterase Drugs.—Segments of guinea-pig ileum, about 3 cm. long, were suspended in a 20 ml. bath containing oxygenated Tyrode solution at 37°. Responses of the preparation to the drugs were recorded by a lever writing on a smoked paper.

After some preliminary experiments with eserine sulphate, neostigmine chloride (Prostigmin chloride) and pyridostigmine bromide (Mestinon, Roche) (Fromherz and Pellmont, 1953; Randall, Conroy, Ferruggia, Kappell, and Knoeppel, 1955), pyridostigmine was selected for use in these experiments because, in concentrations of 10^{-6} in the bath, it gave regular responses, and the intestine relaxed more rapidly after washing than with the other drugs. Anticholinesterases were given every 10 min., and test substances were added 1 min. before.

Peristaltic Reflex.—Segments of intestine about 7 cm. long were suspended in a 25 ml. bath containing oxgyenated Tyrode solution ($MgCl_2$ 0.01 g./l.), according to the method described by Trendelenburg (1917). The peristaltic reflex was elicited by distension of the intestinal lumen by a pressure of 3 cm. of Tyrode solution. The rise in intraluminal pressure was maintained for 1 min. and repeated every 5 min. Test compounds were added to the bath 1 min. before stimulation. Intestinal responses were recorded by two levers, one recording contractions of the longitudinal muscle, and the other the intestinal volume.

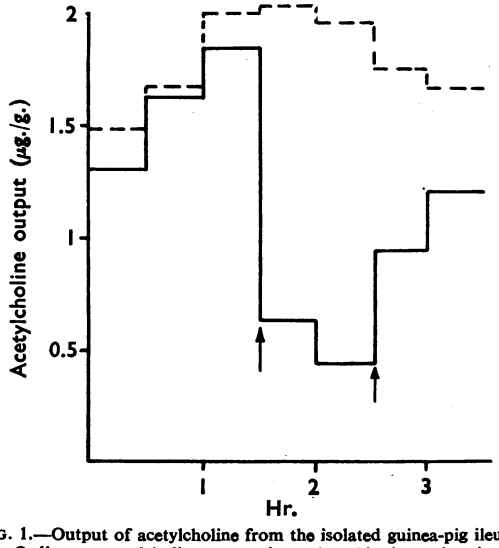

FIG. 1.—Output of acetylcholine from the isolated guinea-pig ileum. Ordinate: acetylcholine output in μg./g. Abscissa: time in hr. Assays of acetylcholine output were made every 30 min. Broken line, control acetylcholine output. Continuous line, acetylcholine output in experiments in which M.G. 1763 was added at the first arrow and the preparation was washed one hour later at the second arrow.

Drugs.—Many acyl-aryl compounds were assayed. This paper reports results obtained with some of the most active substances, namely, diphenylylacetic acid derivatives and diphenylheptylglycollic acid. They were used in the form of the sodium salts.

RESULTS

Action of Sodium Diphenylylbutylacetate (M.G. 1763) on the Output of Acetylcholine from the Isolated Guinea-pig Ileum.—When the acetylcholine release from the intestine in the control samples was high and constant, sodium diphenylylbutylacetate was added to the bath in a concentration of 20 μg./ml. 90 min. after it had been placed in the bath. The results are shown in Fig. 1. The broken line represents average amounts of acetylcholine released from the intestine in 8 control experiments. The continuous line represents the average of 4 experiments with M.G. 1763, added at the first arrow and washed out after 1 hr. Acetylcholine production was much reduced, but recovery was rapid when the drug was washed out.

Effect of Sodium Diphenylylbutylacetate on the Intestinal Responses to Different Anticholinesterase Drugs.—When an anticholinesterase was added to the bath, after a latency of a few seconds contraction of the intestine gradually developed which persisted until the preparation was washed. Larger doses of pyridostigmine were required than of eserine or neostigmine, and the latent period was also a little longer.

Fig. 2 shows the effect of M.G. 1763 (10 μg./ml.) on the intestinal response to eserine (0.5×10^{-6}), neostigmine (0.5×10^{-6}) and pyridostigmine (2×10^{-6}). M.G. 1763 greatly reduced the contractions caused by eserine and neostigmine and completely abolished that caused by pyridostigmine.

Effect of Various Inhibitors of Acetylcholine Synthesis on Intestinal Contractions Produced by Anticholinesterases.—Experiments were next performed to see whether all the compounds inhibiting acetylcholine synthesis *in vitro* were able to antagonize the response of the intestine to anticholinesterases, and whether there was a relationship between activity and chemical structure.

Fig. 3 shows the inhibition by sodium diphenylheptylglycollate (L.10) of the contractions of the isolated guinea-pig ileum induced by pyridostigmine. L.10, which strongly inhibited enzymatic acetylation *in vitro*, is thus very effective in this *in vivo* test.

Various diphenylylacetic acid derivatives were compared for their capacity to reduce the intestinal response to pyridostigmine. The results are summarized in Fig. 4, which shows that increasing length of the side chain can be correlated with increased inhibitory power.

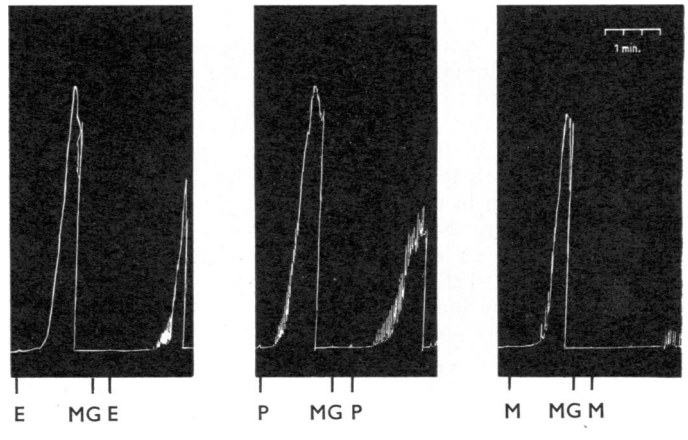

FIG. 2.—The isolated guinea-pig ileum. E, eserine 0.5×10^{-6}; P, neostigmine 0.5×10^{-6}; M, pyridostigmine 2×10^{-6}; MG, diphenylylbutylacetic acid (M.G. 1763) 10 μg./ml. which was added 1 min. before the second dose of each anticholinesterase.

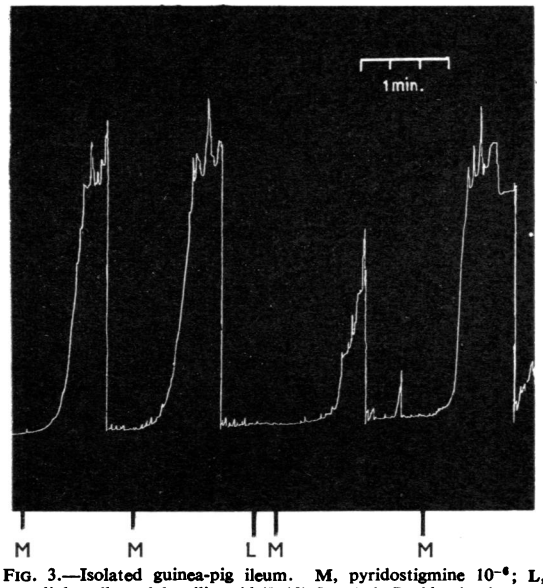

FIG. 3.—Isolated guinea-pig ileum. M, pyridostigmine 10^{-6}; L, diphenylheptylglycollic acid (L.10) 5 μg./ml. Pyridostigmine was added to the bath every 10 min. and allowed to act for 3 min. L.10 was added 1 min. before pyridostigmine.

Comparison of Sodium Diphenylylbutylacetate with Atropine on the Response of the Isolated Guinea-pig Ileum to Acetylcholine and Pyridostigmine.—In order to demonstrate that the compounds tested inhibited intestinal contractions by preventing acetylcholine synthesis and not by antagonizing acetylcholine, they were compared with atropine.

As shown in Fig. 5, M.G. 1763 in a concentration of 100 μg. in 20 ml. scarcely affected the

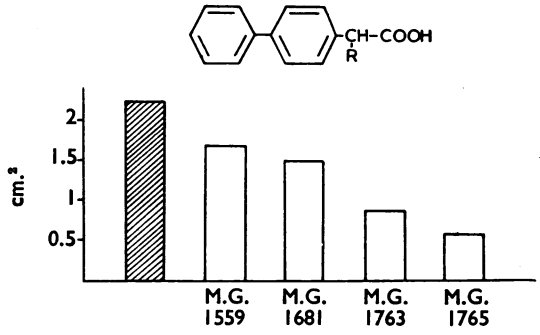

FIG. 4.—Effect of various diphenylylacetic acid derivatives (at a concentration of 10^{-5}) on the responses of isolated guinea-pig ileum to pyridostigmine (10^{-6}, applied for 2·5 min.). The values given are obtained by planimetry of contractions recorded on the tracing. The shaded area represents average value of controls. In the formula above the graph—R in M.G. 1559 is ethyl, in M.G. 1681 is propyl, in M.G. 1763 is butyl and in M.G. 1765 is pentyl.

contraction caused by 0.5 μg. of acetylcholine, but strongly inhibited that caused by 20 μg. of pyridostigmine. Atropine, in a dose of 0.05 μg., which reduced by about 50% height of the contraction caused by the same dose of acetylcholine, scarcely affected the response of the intestine to pyridostigmine.

Effect of Sodium Diphenylylbutylacetate (M.G. 1763) and Sodium Diphenylylethylacetate (M.G. 1559) on the Peristaltic Reflex.—M.G. 1763 inhibited the contraction of the longitudinal muscle responsible for the preparatory phase of peristaltic reflex, but the contractions of the circular muscle associated with the emptying phase were little affected. With increasing doses of

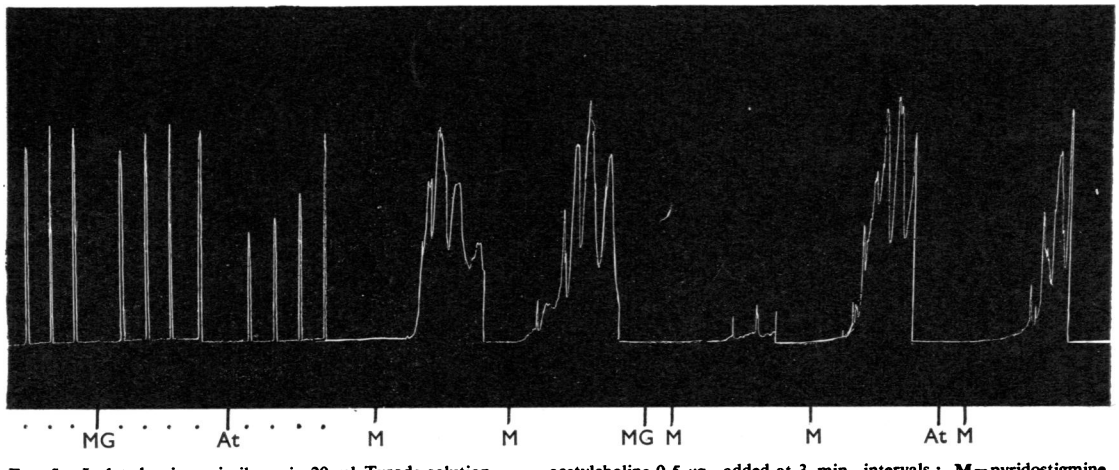

FIG. 5.—Isolated guinea-pig ileum in 20 ml. Tyrode solution. ·,·=acetylcholine 0·5 μg., added at 3 min. intervals; M=pyridostigmine 20 μg. added at 10 min. intervals (contact time 5 min.); MG=diphenylylbutylacetic acid (M.G. 1763) 100 μg. and At=atropine 0·05 μg. Inhibitors were added 1 min. before stimulating drugs.

M.G. 1763 large inhibitions were obtained. The inhibition, which was reversible, could be repeated. M.G. 1559, a compound with a shorter side-chain, was again less effective than M.G. 1763.

Discussion

The results obtained in the present experiments are consistent with the *in vitro* findings, that aryl derivatives of acetic acid are able to inhibit the acetylation of choline.

The output of acetylcholine from the isolated ileum was much reduced by the presence in the bath fluid of diphenylylbutylacetic acid, one of the most powerful of these compounds. At a high concentration of M.G. 1763, the inhibition was complete, but it was always reversed by washing. A similar reduction in acetylcholine output was shown for morphine (Schaumann, 1957), but the inhibition was never complete even at high doses, and morphine was ineffective in inhibiting the synthesis of acetylcholine. The reduction in acetylcholine output produced by morphine was accounted for by a reduced acetylcholine release.

Our compounds inhibited the intestinal contractions produced by anticholinesterase drugs, and this inhibition does not seem to occur by an atropine-like mechanism, since the responses to acetylcholine were not or only slightly affected by atropine; neither was there any *in vitro* inhibition of anticholinesterase activity (Morpurgo, unpublished observation).

In the Trendelenburg preparation, our compounds were found to inhibit the longitudinal contraction of the ileum, while the peristalsis was scarcely affected. Explanation of this observation is difficult because the mechanisms involved in the different phases of the peristaltic reflex are not yet fully ascertained. The effects of different inhibitors have been examined (Feldberg and Lin, 1949; Kosterlitz and Robinson, 1957; Schaumann, 1955). Thus local anaesthetics and ganglion-blocking agents inhibit the circular contractions of the emptying phase, showing its nervous origin, while the contractions of the longitudinal muscle persist, probably because they are myogenic. Atropine and morphine affect both preparatory and emptying phases. As to the inhibition of longitudinal contractions exerted by our compounds, they may prevent the synthesis of acetylcholine by the intestinal muscle, since it has been shown that the acetylcholine in the intestinal wall is largely synthesized by muscle and not by nervous structures (Feldberg, 1949).

A relationship between the inhibitory activity on intestinal contractions and chemical structure was also shown by these as well as by the *in vitro* experiments. This supports the hypothesis that the phenomenon is due to the inhibition of acetylcholine synthesis.

Diphenylylacetic acid derivatives (M.G. compounds) were synthesized in Maggioni Laboratories, Milan (Cavallini, Massarani, Nardi, and Ambrosio, 1957), and diphenylheptylglycollic acid (compound L.10) was supplied by Carani in Lofarma Laboratories, Milan.

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