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SULFUR AND SELENIUM ISOLOGS RELATED TO ACETYLCHOLINE AND CHOLINE—IX

FURTHER COMPARATIVE STUDIES OF THE PHARMACOLOGICAL EFFECTS OF ACETYLCHOLINE AND ITS THIO AND SELENO ANALOGS AND THEIR HYDROLYSIS PRODUCTS*

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Abstract—The pharmacological effects of acetylthiolcholine (ASCh) and acetylselenolcholine have been compared with those of their oxygen analog, acetylcholine, on the guinea pig ileum, innervated rat diaphragm, and rat blood pressure preparations. The close similarity of the affinity constants for the muscarinic antagonist, lachesine, obtained with all three members of the series, is interpreted as evidence that they act primarily on the same receptor system. Further evidence is offered to support the contention that the unusual actions of these thio and seleno analogs of acetylcholine (ACh) on the blood pressure and at the neuromuscular junction, are attributable to the relatively high activity of their hydrolysis products which readily undergo oxidation to *bis*-onium compounds of lesser activity. This unusual chemical behaviour provides possible explanations for further recently reported anomalies in the pharmacology of ASCh.

IN A PREVIOUS comparative study[1] of some of the pharmacological effects of ACh and its thio and seleno analogs (Fig. 1), it was shown that many of the reported anomalous

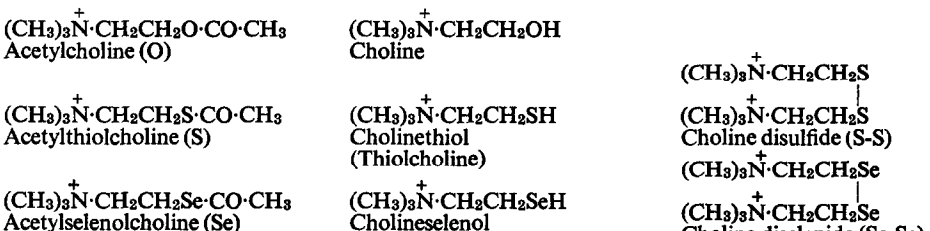

FIG. 1. Structural formulae of thio and seleno analogs of choline and acetylcholine.

actions of ASCh[2–6] could be rationalized on the basis of differences in the biological activities of the thiolester and selenolester, their hydrolysis products, and the oxidation products of the latter. Although basically similar, the pattern of activity displayed by acetylthiolcholine was accentuated in the case of acetylselenol-choline.

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One rather unexpected observation regarding the pharmacology of ASCh, which could not be explained on the basis of its chemical behaviour, was that reported in 1960 by Wurzel.[7] In this paper, the activity of ASCh on the isolated guinea pig ileum preparation was compared with that of ACh, and the effect of atropine in modifying the responses evoked by both esters was investigated. Wurzel reported an equipotent molar ratio of 60–90 for ASCh, relative to ACh* and then stated that ". . . although atropine sulfate 0·1 μg/ml in the Tyrode solution antagonizes choline esters very efficiently, it was practically ineffective on the contraction of the guinea pig intestine caused by thiolcholine esters . . ." This finding led Wurzel to propose that "thiolcholine esters are devoid of muscarinic potency".[9]

During our previous study, it had been assumed, solely as a working hypothesis which remained to be tested, that the primary effects of both ASCh and acetylselenolcholine on the ileum preparation were mediated, like ACh, via the muscarinic receptors.† Wurzel's reported lack of effect of atropine would preclude such an assumption and, in addition to repeating his experiment, we have attempted to investigate this point in a more specific and quantitative manner by determining the affinity constant (K_b) for a specific muscarinic antagonist (lachesine),[11, 12] employing each of the three esters in turn as agonist in the determination. Lachesine (Ethyl(2-hydroxyethyl) dimethylammonium benzilate chloride) was selected as the standard muscarinic antagonist for two reasons. First, it is generally regarded as having a somewhat higher specificity for muscarinic receptors than does atropine, and second, it has the merit of being a quaternary ammonium compound, as are all the agonists which were used in conjunction with it. The antagonism produced by lachesine will therefore be restricted to the superficial muscarinic receptors upon which the agonists will exert their effects, whereas with atropine it would be possible for the antagonist to penetrate to sites to which the exogenously applied quaternary agonists could have no access.

The original investigation of this series of isologous compounds was restricted to a quantitative examination of their effects on intestinal smooth muscle and on the slow-fibre, skeletal muscle of the frog rectus abdominis. In view of the fact that the oxidation products of both cholinethiol and cholineselenol are *bis*-onium compounds, it seemed a logical extension of the work[13–15] to study the effects of these substances and of the original esters, specifically at the neuromuscular junction. The results reported

* The equipotent molar ratio for ASCh obtained by Wurzel is significantly less than the value we have obtained (320). Such a difference cannot be accounted for entirely on the basis of different experimental procedures. We have noticed, however, that certain batches of commercially obtained ASCh do show a much higher activity than that normally found. Since two-dimensional paper chromatography of all commercial samples that we used showed the presence of at least two components, it seems likely that the reported high activity is due to the presence of a small amount of a more active material. Such a variation from batch to batch has already been noted by Ellman, as quoted in a recent paper by Salafsky.[8] It is possible that the contaminant may be the methyl ether of thiolcholine ($CH_3S \cdot CH_2CH_2NMe_3$), which we have found to have approximately twice the activity, on the ileum preparation, of ASCh.

† It has not, of course, been adequately demonstrated that, on such a preparation, the effect of ACh is produced solely by its interaction with a single species of ("muscarinic") receptors.[10] The above reference to ACh's effects being mediated "via the muscarinic receptor" must therefore be regarded as a restatement of a generally accepted convention with no implication that such an assumption has been shown unequivocally to be true.

in this paper were obtained on the isolated hemidiaphragm preparation of the rat, indirectly stimulated via the phrenic nerve.

Since most of the earlier work on the pharmacology of ASCh, from which so many apparent contradictions arose, was concerned with its effects on the blood pressure of various species, we have repeated some of the experiments in an attempt to rationalize such observations in the light of the rather unusual chemical behaviour of these ACh analogs.

EXPERIMENTAL

Materials

Acetylcholine chloride was purchased from Merck, acetylthiolcholine iodide from Nutritional Biochemicals Corp., and neostigmine (Prostigmin methylsulfate) from Roche. Acetylselenolcholine bromide and *bis*(2-trimethylammoniummethyl) diselenide diiodide were kindly supplied by Dr. W. H. H. Gunther. *Bis*(2-trimethylammonium-methyl)disulfide diiodide, m.p. 250°, was prepared by quaternization of the corresponding tertiary aminodisulfide with methyl iodide with methylethyl ketone as solvent.

Preparations

Guinea pig ileum. The preparation was set up as before.[1] Determinations of antagonist affinity constants were made by the procedure previously described by Barlow, Scott and Stephenson.[16]

Innervated rat diaphragm. The preparation described by Bülbring[17] was used. The muscle was mounted in a 20-ml bath containing McEwan's solution[18] maintained at 37° ± 0·2° and aerated with 95% oxygen and 5% carbon dioxide. The phrenic nerve was stimulated by supramaximal rectangular pulses, 0·3 msec in duration applied at a frequency of 6/min, and muscle twitches were recorded by a semi-isometric spring-loaded lever writing on a smoked drum.

Rat blood pressure. Male rats (200–300 g) were anesthetized with chloralose (1%)–urethane (10%) given by i.p. injection. Blood pressure was recorded from the carotid artery with a Statham pressure transducer. All drugs were administered in a volume of 0·1 ml by injection into the jugular vein.

RESULTS

Effects of muscarinic antagonists

Atropine. The presence of hexamethonium (50 mg of the bromide/l.) reduces to a certain extent the activity of the thiol and selenol esters on the guinea pig ileum preparation, presumably by blocking effects mediated via the parasympathetic ganglia.[19] In order to restrict the analysis to a comparison of the direct muscarinic effects of the three esters, the above concentration of hexamethonium was incorporated into all Tyrode solutions employed in the ileum experiments. Although their dose-response curves are essentially parallel, the nature of the response produced by each ester differs slightly; thus with ACh the muscle contracts smoothly and regularly and the contraction is usually maintained at a steady value throughout the 10-sec period of contact with the drug solution. With the thiol ester, the muscle contraction follows roughly the same time course as with ACh but, once the contraction has reached its plateau level, there is a tendency for the ileum to exhibit rapid, low-amplitude, contraction-relaxation oscillations, giving rise to a serious of small 'spikes' on the trace.

This tendency is even more evident with the selenol ester and, in describing the

effects of antagonists on the smooth muscle contractions produced by these drugs, care must be taken to distinguish between these two types of response.

In addition to blocking the action of ACh (4×10^{-8} M), the concentration of atropine employed by Wurzel[7] (0·1 μg atropine sulfate/ml; equivalent to 3×10^{-7}M with respect to atropine) was found by us to abolish completely the effects, on the ileum preparation, of 4×10^{-5} M ASCh, which when administered before the atropine, evoked a near maximal response. With slightly higher doses of ASCh the main contraction of the muscle was effectively prevented by the same concentration of atropine but the 'spike' activity, which previously had been observed during the sustained contraction to this drug, was not entirely abolished by the antagonist. Acetylselenolcholine behaved in a similar manner, the 'spike' activity being more noticeable.

Lachesine. A typical kymograph tracing obtained in the determination of the affinity constant (Kb) for lachesine is shown in Fig. 2. The figures before the arrow

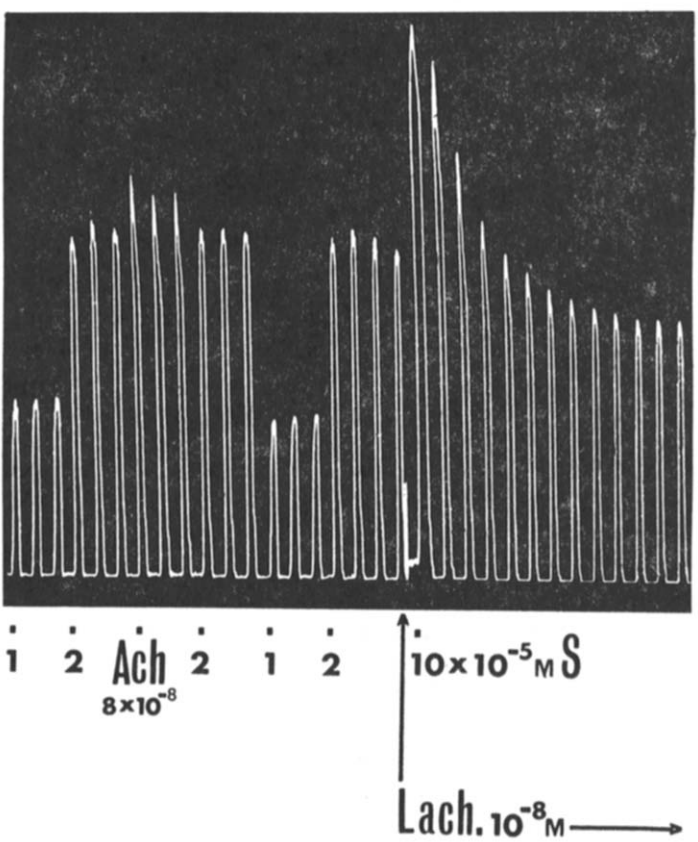


FIG. 2. Determination of lachesine affinity constant with acetylthiolcholine (S) as agonist. Figures refer to molar concentrations of S ($\times 10^{-5}$) with exception of the trio of acetylcholine (ACh) doses. Guinea pig ileum preparation: drug contact time = 10 sec. Cycle = 90 sec, load = 0·5 g, magnification $\times 4$.

refer to concentrations ($\times 10^{-5}$ M) of the agonist, acetylthiolcholine (S), to which the ileum was exposed in the absence of the antagonist. At 'Ach,' three successive doses

of 8×10^{-8} M Ach were given for purposes of comparison. At the arrow, the bathing fluid was replaced by a 10^{-8} M solution of lachesine in Tyrode and subsequent doses of the agonist contained 10^{-4} M ASCh together with 10^{-8} M lachesine. The affinity constant was calculated as previously described.[16]

TABLE 1. ANTAGONIST AFFINITY CONSTANTS FOR LACHESINE WITH ACETYLCHOLINE (O), ACETYLTHIOLCHOLINE (S) AND ACETYLSELENOLCHOLINE (Se) AS AGONIST

		Lachesine K_b	
Compound	EPMR*	Individual K_b values	Mean K_b (± S.E.)
Acetylcholine	1	8·64 8·88 × 10^8 8·40 8·36	8·6 (± 0·10) × 10^8
Acetylthiolcholine	320	4·9 6·7 × 10^8 6·0 5·6	5·8 (± 0·33) × 10^8
Acetylselenolcholine	220	8·1 7·3 × 10^8 7·9	7·8 (± 0·20) × 10^8

* Values of equipotent molar ratios (EPMR) are relative to acetylcholine (ACh = 1) and are taken from previously published work.[1]

The second column of Table 1 shows the Kb values obtained for lachesine by using each of the three esters as agonist. The first column gives, for comparison, the values of the equipotent molar ratios for the three esters previously reported.[1]

Effects at the neuromuscular Junction

Preliminary attempts were made to assess the activities of the compounds on the isolated rat diaphragm preparation by determining the minimum concentration required to produce a slight but measurable enhancement of the muscle twitch evoked by stimulation of the phrenic nerve. The degree of potentiation so produced was very slight, due partly to the necessity of stimulating the nerve supramaximally in order to obtain a constant twitch height. The extent of the enhancement of the response was further limited by the fact that only a small increase in the dose readily produced a block of neuromuscular transmission. On the basis of their ability to produce a slight increase in twitch heights, acetylthiolcholine (S) and acetylselenolcholine (Se) are approximately equiactive and are some 40 times more active than acetylcholine (O). Remarkably consistent values for such activity were obtained on a series of different rat diaphragm preparations despite the fact that no eserine or neostigmine was present to prevent the effects of cholinesterase on the esters.

Fig. 3 shows the effects of neostigmine on concentrations ($\times 10^{-4}$ M) of the three esters which were equiactive in producing neuromuscular block in the isolated rat diaphragm preparation. At the arrow, the bathing fluid was replaced by one containing neostigmine (250 μg Prostigmin methylsulfate/l.) and stimulation of the preparation was continued at the standard rate (f = 0·1) for 30 min before addition of any other drugs.

The responses of the preparation, equilibrated with neostigmine in the manner described, to the same doses of acetylthiolcholine (S) and acetylselenolcholine (Se),

given before the addition of neostigmine, were either unaffected or slightly reduced, while the responses of acetylcholine (O) were markedly enhanced. In the particular experiment illustrated, approximately 1/30 of the dose of ACh given before neostigmine was sufficient to produce an almost complete neuromuscular block in the presence of the anticholinesterase.

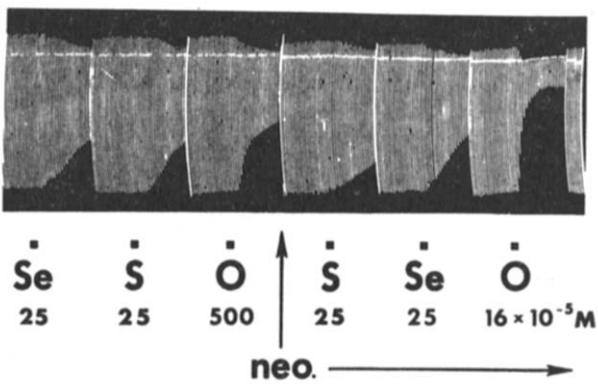


FIG. 3. Rat diaphragm preparation. Comparison of effects of neostigmine on concentrations of esters producing approximately the same degree of neuro-muscular block. At 'neo' kymograph stopped, bath fluid was replaced by one containing $7{\cdot}5 \times 10^{-7}$ M neostigmine, and preparation was allowed to stabilize for 30 min while stimulation continued at standard rate ($f = 0{\cdot}1$ pulse. sec^{-1}). Figures refer to bath concentrations $\times 10^{-5}$ M.

In most cases it was found that the continued presence of neostigmine reduced the response to either ASCh or acetylselenolcholine; in addition, it altered the time course of the development of the neuromuscular block.

It can be seen from Figs. 4 and 5 that the block appears to develop more slowly in the absence of neostigmine than when it is present. Furthermore, in the absence of neostigmine, the block slowly increases on continued exposure to the drug, whereas

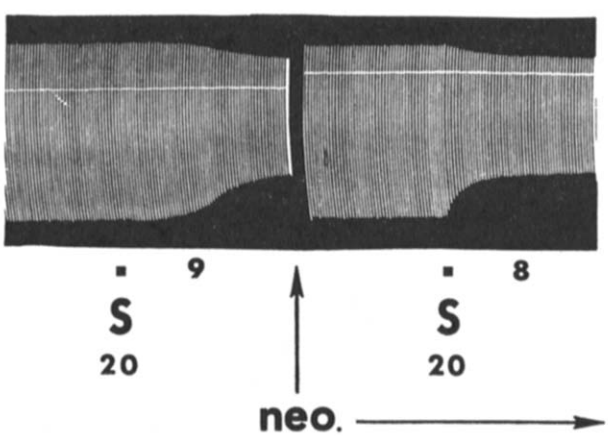


FIG. 4. Rat diaphragm preparation. Effect of neostigmine on neuromuscular block produced by acetylthiolcholine (S). Figures refer to bath concentration $\times 10^{-5}$. Numbers after dots give drug contact time (min). At 'neo', preparation equilibrated with $7{\cdot}5 \times 10^{-7}$ M neostigmine for 30 min as before.

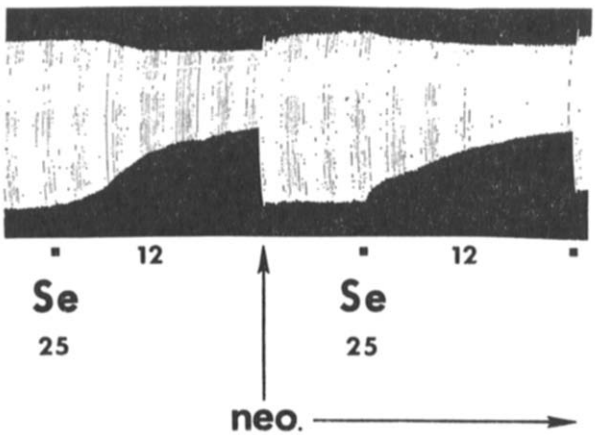


FIG. 5. Rat diaphragm preparation. Effect of neostigmine on neuromuscular block produced by acetylselenolcholine (Se). Figures refer to bath concentration $\times 10^{-5}$. Numbers after dots give drug contact time (min). At 'neo', preparation equilibrated with $7{\cdot}5 \times 10^{-7}$ M neostigmine for 30 min.

in the presence of neostigmine a stable block can be established fairly readily; this is particularly evident in Fig. 4. As might be anticipated, both the disulfide (S-S) and the diselenide (Se-Se) produce a depolarizing type of neuromuscular block which is potentiated by neostigmine (Figs. 6 and 7). It is interesting to note that in the electroplax preparation the disulfide and diselenide exert an anti-depolarizing block.[20]

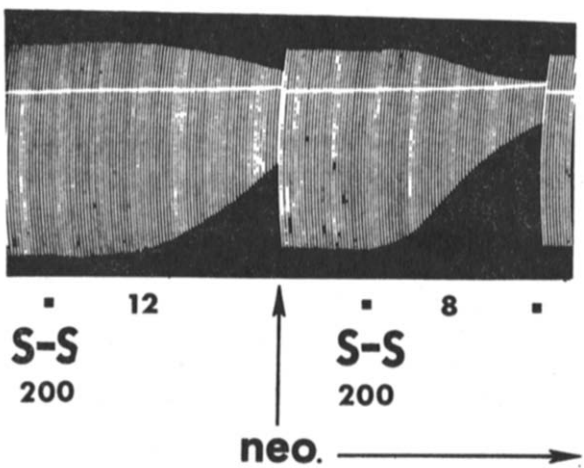


FIG. 6. Rat diaphragm preparation. Effect of neostigmine on neuromuscular block produced by choline disulfide (S-S). Figures refer to bath concentration $\times 10^{-5}$. Numbers after dots give drug contact time (min). At 'neo', preparation equilibrated with $7{\cdot}5 \times 10^{-7}$ M neostigmine for 30 min as before.

Effects on blood pressure

The i.v. injection of ACh into the rat causes only a fall in blood pressure in the absence of atropine.[21] With increasing doses of ACh, a lowering of heart rate also becomes evident, but it is only when atropine has been given before the ACh that the pressor (nicotinic) effect of the latter can convincingly be demonstrated.

Fig. 8 shows the effects on the blood pressure of the rat of two dose levels of ASCh in the absence of atropine and in the presence of neostigmine (Prostigmin methylsulfate, 120 μg/kg). The immediate response to an injection of the thiolester was a fairly rapid fall in blood pressure similar to that produced by ACh, although not

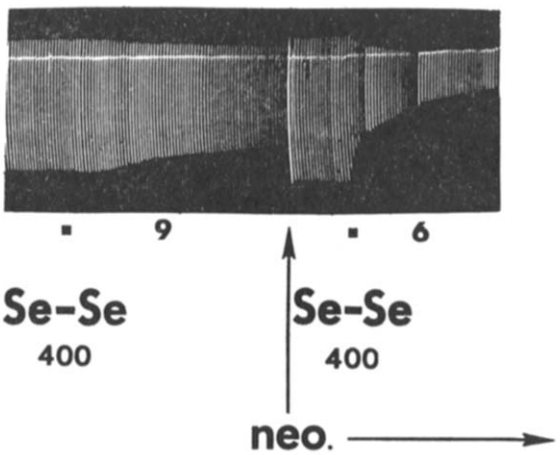


FIG. 7. Rat diaphragm preparation. Effect of neostigmine on neuromuscular block produced by choline diselenide (Se-Se). Figures refer to bath concentration $\times 10^{-5}$. Numbers after dots give drug contact time (min). At 'neo', preparation equilibrated with $7{\cdot}5 \times 10^{-7}$ M neostigmine for 30 min as before.

as marked. After the initial depressor response (−35 mm Hg) to ACh, the blood pressure gradually returned to normal, but with ASCh the initial fall in blood pressure (−15 mm Hg) was followed immediately by a pressor response of some 10 mm Hg, after which the blood pressure returned to normal within 1·5 min. Increasing the dose of ASCh 5-fold did not greatly affect the magnitude of the initial depressor response, although it made it more transient, but it did produce a marked potentiation of the subsequent pressor effect (+40 mm Hg) and revealed a sustained secondary pressure rise after the main pressor response had subsided. Under similar experimental conditions, the effects of acetylselenolcholine were similar to those of its sulfur analog, except that the depressor response was generally smaller and of shorter duration and the pressor effect was more pronounced and prolonged.

It has been demonstrated by many workers that the effects of ACh on the blood pressure are almost exclusively due to the ester itself and not to the choline liberated on its hydrolysis. Previous work has shown clearly that such an assumption may not be made in the case of the ASCh or acetylselenolcholine and the remainder of the experiments reported in this paper were concerned with elucidating the contributions of the various transformation products to the effects on blood pressure after i.v. injection of the esters.

Fig. 9 shows one such comparative study in which biologically equivalent amounts of the ester alone, the prehydrolyzed ester, and the final oxidation product were injected in the absence of both atropine and neostigmine. That the conditions of hydrolysis by isolated acetylcholinesterase were sufficient to ensure complete destruction of the ester is demonstrated by the second group of tracings (ACh).

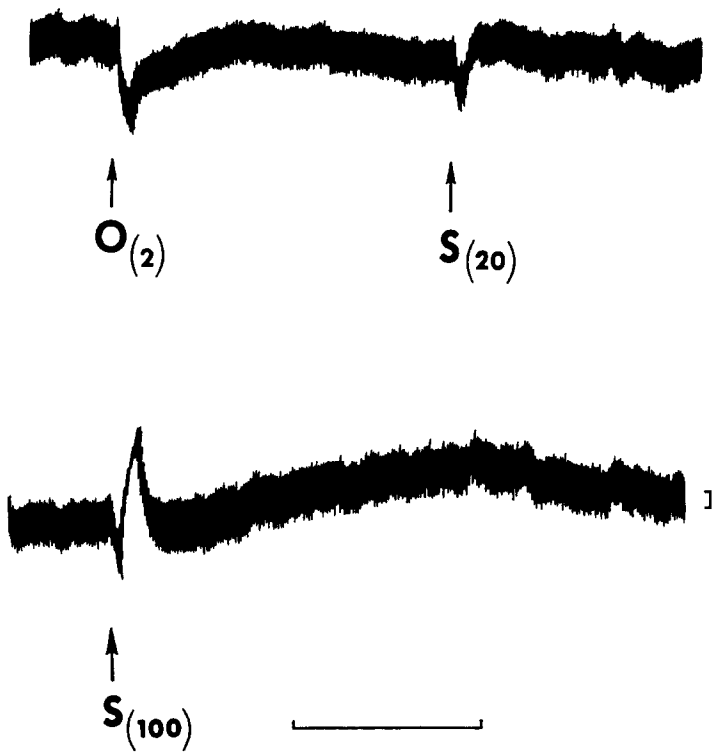


FIG. 8. Rat blood pressure. Responses to i.v. injection of acetylcholine (O) and acetylthiolcholine (S). No atropine present. Neostigmine (120 μg methylsulfate/kg) given i.v. infusion 5 min before injection of drugs. Figures refer to nanomoles of ester injected. Vertical calibration, 10 mm Hg; horizontal calibration, 2 min.

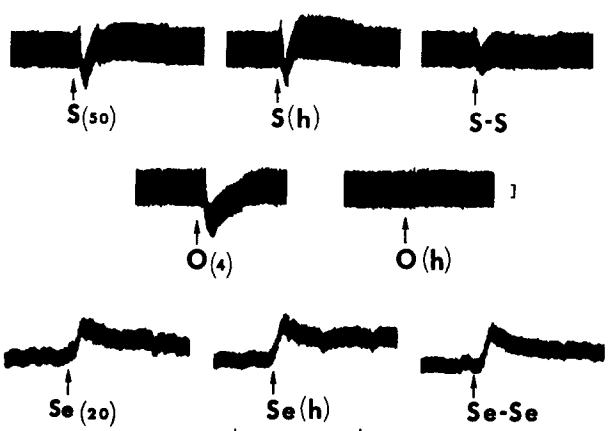


FIG. 9. Rat blood pressure. No neostigmine given. Comparative effects of esters (O, S and Se), prehydrolyzed esters (h), and final oxidation products (S-S and Se-Se). Figures refer to nanomoles of injected drug. Vertical calibration, 10 mm Hg; horizontal calibration, 2 min.

Hydrolysis of the ASCh before injection leads to a slight reduction in the depressor response, but almost doubles the pressor response (+15 mm in contrast to +8 mm) and markedly increases the duration of the effect. At the equivalent dose level used, the depressor effect of the disulfide (S-S) was very small and it produced no detectable pressor response.

The lower series of blood pressure tracings in Fig. 9 shows the comparative effects of the ester, the selenol (undoubtedly with at least a portion of the molecules in the diselenide form) and the authentic diselenide (Se-Se), when injected in equivalent amounts. In this particular instance no depressor effect was detected in response to any of these substances. As mentioned earlier, the depressor response to acetylselenolcholine tended to be smaller and more fleeting than that evoked by the thiolester and, although some such effect was observed on most preparations, it was found to be very dependent on the rate of injection (although not an injection artifact) and was usually absent when the ester was given by slow injection.

It can be seen readily that prior hydrolysis of the selenol ester increases both the magnitude and the rate of onset of the pressor response as well as prolonging its duration. The increase in blood pressure produced in this particular experiment by the diselenide alone, proved to be somewhat greater than that usually seen with this compound.

Fig. 10 shows the comparative effects of the three esters before and after the administration of neostigmine (80 μg neostigmine methylsulfate/kg). Prevention, or at least reduction of the hydrolytic effects of cholinesterase resulted in the usual markedly enhanced depressor response to ACh and greatly extended the time taken for the blood pressure to return to the normal level. When preserved in the form of the ester, by the continued presence of neostigmine, ASCh shows a much closer resemblance to its oxygen analog, with a greatly increased depressor effect followed by a much reduced and less prolonged pressor response.

With the selenol ester, the depressor effect was virtually unchanged after neostigmine, but the subsequent rise was noticeably less than when the ester was subject to enzymatic hydrolysis. The return of the elevated blood pressure to the normal level was

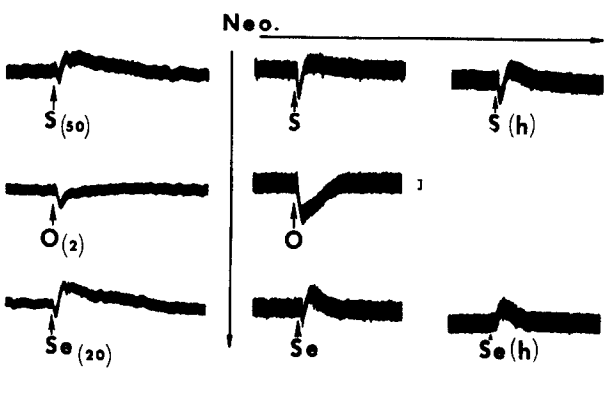


FIG. 10. Rat blood pressure. Comparative effects of esters before and after administration of neostigmine. At 'neo', 80 μg neostigmine methylsulfate/kg given by slow injection. Figures refer to nanomoles of injected drug; (h) denotes ester was subjected to hydrolysis by acetylcholinesterase immediately prior to injection. Vertical calibration, 10 mm Hg; horizontal calibration, 2 min.

also accomplished in a much shorter time.

Prior enzymic hydrolysis of the thiol and selenol esters produced essentially the same effect, viz. a reduction in the depressor response (disappearing altogether in the case of the acetylselenolcholine), a potentiation of the secondary rise in blood pressure followed by a more rapid return to the normal level.

In Fig. 11 the effects on the rat blood pressure of increasing doses of the *bis*-onium disulfide (S-S) and diselenide (Se-Se) are illustrated. The doses used to produce such effects are much higher than those which could arise from the complete conversion by hydrolysis and oxidation of the doses of esters employed in the previous experiments (Figs. 8–10).

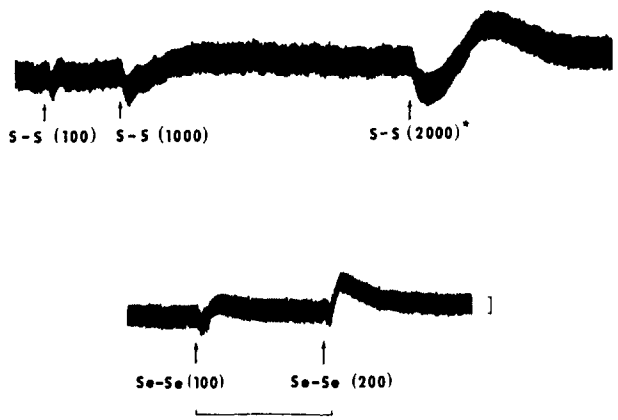


FIG. 11. Rat blood pressure. Effects of large doses of *bis*-onium compounds in presence of neostigmine (80 μg neostigmine methylsulfate/kg, given 5 minutes before injection of first drug). Vertical calibration, 10 mm Hg; horizontal calibration, 2 min. ★This dose given in a volume of 0·2 ml by slow injection.

DISCUSSION

Wurzel[7] has suggested "that the contraction of the intestine (guinea pig) in response to thiolcholine esters (specifically, the acetyl, propionyl, butyryl and benzoyl esters) be termed a nicotinic effect". This suggestion is based primarily on his observations that (1) the "order of potency" of the thiolcholine esters, determined on the isolated guinea pig intestine preparation, is similar to the "nicotinic order of choline esters" as determined on "the frog rectus muscle, the cat (atropinized) blood pressure rise, and the eserinized rat diaphragm preparation"; and (2) the effect of thiolcholine esters was "insensitive to atropine."

It is not stated what drug contact time was employed in these ileum experiments and hence it is impossible to deduce the extent, if any, of ester hydrolysis which might have occurred during the period the drug was in contact with the preparation. Koelle[22] found that, although both the butyryl and benzoyl esters of cholinethiol are resistant to hydrolysis by acetylcholinesterase, they are hydrolyzed by pseudocholinesterase, and several workers[23–25] have reported that pseudocholinesterase is the predominant cholinesterase in the intestine and that it is present in large quantities. In view of this, one is tempted to advance the hypothesis that the order of activity of the three aliphatic esters might be correlated with their quoted rates of hydrolysis

by pseudocholinesterase if one assumes that the activity is due almost entirely to cholinethiol itself, the common product of such a reaction. This, of course, does not take into account any contribution to the overall activity by the esters themselves. However, on the basis of the data reported in the above paper,[7] benzoylthiolcholine does not fit into the proposed scheme, although in ours hands the activity of benzoylthiolcholine on the isolated ileum preparation is more than half that of ASCh and not one-sixth, as reported by Wurzel.

The results given in Table 1 indicate that, particularly when effects arising from stimulation of parasympathetic ganglia are eliminated, the actions of the three esters on the isolated guinea pig ileum are competitively antagonized by the specific muscarinic antagonist, lachesine. A not unreasonable deduction from these results would be that, in evoking contractions on the guinea pig ileum preparation, the three esters all act on the same receptor, namely the 'muscarinic' receptor insofar as this has been defined.

In comparing the effects of the ester series on the isolated rat diaphragm preparation with those previously obtained on the guinea pig ileum, certain pertinent intrinsic differences must be borne in mind. First, in the rat diaphragm preparation the access of the drug to the receptor is limited by diffusion, whereas in the ileum the diffusion limitation might well be regarded as negligible.[26] As a consequence of the diffusion factor, it is necessary to expose the muscle to the drug for a much longer time in the case of the diaphragm (at least 3 min) than with the ileum (10 sec). This means that the opportunity for enzymatic modification of the drug molecule, while in the process of diffusing to the site where it acts, is much greater with a tissue of more than limited thickness (as with the diaphragm) than with one where the receptors appear to be located on or near the surface of the muscle (e.g. ileum).

From our observations on the rate of onset of neuromuscular block in the diaphragm preparation, it was evident that the rates of penetration of the esters, the cholines, and the *bis*-onium compounds were quite different. The esters appear to penetrate quite as readily as ACh, whereas the disulfide and diselenide are able to diffuse only slowly. Because of this diffusion difference, the drug could penetrate toward the receptor sites in the form of the ester and, when through at least some of the barriers, could be converted by enzymatic hydrolysis and subsequent oxidation to the corresponding *bis*-onium compound. In this manner a concentration of the *bis*-onium compound could be built up at the receptor from a relatively small bath concentration of the ester. Such a gradual onset of block, in fact, was observed with low concentrations of both the thiol and selenol esters and, once established, the block persisted for a relatively long time after removal of the drug solution from the bath.

Care must also be taken in comparing results obtained previously on the frog rectus preparation with those from the diaphragm presented in the preceding section. Both preparations are 'nicotinic' in character; both require considerable penetration of the drug before a response is manifest;[26] and both are relatively rich in cholinesterase. However, in the case of the rectus, the only agonist present is the added drug (plus its transformation products), whereas in the diaphragm at least two agonists are competing for the same receptor, viz. the added drug (plus products) and the endogenous ACh liberated by stimulation of the phrenic nerve.

It is fairly evident that the presence of neostigmine greatly potentiates the blocking

effects of ACh (Fig. 3), but it has either no effect on those of ASCh or acetylselenolcholine or it antagonizes them to a small extent. By 'equilibrating' the preparation with neostigmine in the manner described, we have attempted to minimize the interference due to its direct cholinomimetic action, which, if not eliminated, was at least held constant throughout this part of the experiment.

Usually one sees a definite change in the rate of onset of block (Figs. 4 and 5) produced by the thiol and selenol ester after the preparation has been treated with neostigmine. This observation is consistent with the idea that, after neostigmine treatment, only one molecular species is responsible for the recorded effect, namely the rapidly penetrating ester. In the absence of neostigmine, the biophase concentration of the ester, which is undoubtedly a more active depolarizing-type blocking agent than the corresponding *bis*-onium compound, rises only slowly due to the action of the cholinesterase to which the ester is exposed en route to the receptor. However, as the biophase concentration of the *bis*-onium compound builds up, the block slowly increases; it is almost impossible to obtain a stable degree of block in the absence of neostigmine and quite easy to do so when it is present (Fig. 4). The simplest explanation for the failure of neostigmine to potentiate the block produced by the thiol and selenol esters is that, as with the ileum and the rectus, the immediate product of enzymatic hydrolysis, the thiol or selenol, is a more effective depolarizing agent than the ester from which it is derived.[20] As might be anticipated from molecular structure considerations, both the disulfide and the diselenide produce a depolarizing type of block, on the isolated rat diaphragm preparation, which is potentiated by the presence of neostigmine.

In a recent paper by Wurzel, Efird and Goldberg,[4] some observations are reported on the effects of ASCh on both the cat superior cervical ganglion and the isolated rat diaphragm preparation. These authors reported that "on the cat superior cervical ganglion ASCh (acetylthiolcholine) is antagonized by prostigmine," an effect which was different from that anticipated and for which they could offer no explanation.

We suggest that the observed reduction in activity of the thiolcholine esters on ganglia by neostigmine is a further consequence of the unexpectedly high activity of the cholinethiol. Thus, when the drug is administered as the ester, at least part of it becomes hydrolyzed to the more potent cholinethiol before acting on the ganglionic receptors. Prevention of this hydrolytic activation step by inhibition of the cholinesterase will give rise to an apparent decrease in the activity of the original drug.

The results reported herein do not support the statement "that (the effect of) ASCh is similarly (to ACh) potentiated by neostigmine on the rat nerve-muscle preparation."[4] Certainly the decrease in ASCh block after neostigmine was never observed to equal that seen with the frog rectus preparation,[1] but in view of the essential differences between these two preparations (*vide supra*) such a result is not altogether surprising. What can be stated with certainty is that, compared to the very marked enhancement of the ACh block produced by neostigmine, its effect on the ASCh and acetylselenolcholine block is negligible.

Reitzel and Long[5] have also reported an apparently unexpected effect of ASCh on the neuromuscular junction. They tested a large series of compounds chemically related to choline "for their ability to antagonize the HC-3 (hemicholinium) induced neuromuscular blockade" of the rabbit sciatic nerve–gastrocnemius muscle preparation. They noted that "in higher doses" ASCh intensified the neuromuscular blockade

produced by HC-3 and observed none of the expected antagonism with this thiol ester. Under the conditions employed, choline continued to be an effective antagonist. Reitzel and Long suggested that "in order for a choline ester to antagonize HC-3, hydrolysis of that ester to choline is necessary," but drew attention to the fact that although ASCh was hydrolyzed by cholinesterase, it was not capable of antagonizing HC-3. The above observation can most readily be explained by the assumption that, with the dose levels and conditions employed, the hydrolyzed ASCh was almost completely converted to the disulfide which, being a *bis*-onium neuromuscular blocking agent, would be expected to intensify the neuromuscular blockade.

The results shown in Fig. 8 confirm the observations of earlier workers[6] in demonstrating that, even in the absence of atropine, ASCh produces both depressor and pressor responses after i.v. injection. The relatively large amount of neostigmine present in the animal used for this experiment makes it very likely that the recorded effects were essentially those of the ester itself, unaffected by enzymatic hydrolysis.

It is fairly evident that, in the concentrations which could arise from the doses of esters administered, the disulfide (and probably the diselenide as well) contributes little to the over-all effects on the blood pressure.

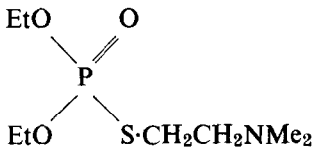
Enzymatic (AChE) hydrolysis *in vitro* of both the thiol and selenol ester immediately prior to injection results in a potentiation of the pressor effect. It therefore seems likely that at least part of the pressor activity of ASCh (and acetylselenolcholine) should rightly be attributed to the corresponding thiol (or selenol). This view is substantiated by the comparative experiments shown in Fig. 10.

With increasing doses of both *bis*-onium compounds, there is a tendency for the pressor effect to become more pronounced, while the depressor effect is diminished or remains unaltered. This indicates that, despite the apparent molecular similarity, the disulfide and diselenide do not share the activity of hexamethonium in lowering the blood pressure.

In a recent paper on the ganglionic stimulating properties of choline and thiolcholine esters, Gebber and Volle[27] described a pattern of activity for the thiolesters which is similar to that reported previously[1] and also discussed above. They found that the esters they employed (ASCh, butyrylthiolcholine, and acetyl-β-methylthiolcholine) were considerably more effective than their oxygen analogs in evoking post ganglionic firing and ganglionic depolarization on the superior cervical ganglion of the cat, and that the responses which they produced were slower in onset, of greater duration and were either unaffected or reduced by the presence of an anticholinesterase. Similar observations had been noted by Liljestrand and Zottermann[3] on the firing in afferent nerves from the carotid body in the cat.

The authors point out that, with the exception of the latency of the response to thiolcholine esters, which was not altered by the presence of an anticholinesterase, their observations are in accord with our earlier suggestion, that the activity of the hydrolysis products of ASCh is responsible for its unusual pharmacology. However, they regard the delayed responses as "an inexplicable characteristic of thiolcholine substances."

It is difficult to form any definite opinion regarding the effects of eserine on the action of the thiolesters, since experiments in which this was employed as the cholinesterase inhibitor were not reported in detail; in most of the experiments the substance 217 AO was employed:



This type of organophosphorus anticholinesterase was first described by Ghosh and Newman[28] and subsequently employed by Koelle and Steiner[29] in their studies on the central effects of anticholinesterases. Gebber and Volle acknowledged that the "blocking action of 217 AO must be regarded as extremely unusual" and gave other evidence to indicate that in many respects its behavior as an anticholinesterase is atypical, particularly in its effects on the responses to the thiolesters. It is possible that part, at least, of the unusual behavior could be explained on the basis of the observations reported by Tammelin,[30] who found that such compounds were in general potent cholinesterase inhibitors and that they acted by phosphorylating the enzyme with release of thiolcholine or the dimethylaminoethylthiol analog. The 217 AO compound would of course be expected to release dimethylaminoethanethiol and not cholinethiol but, in view of the evidence presented by Tammelin to indicate that "the electronic distribution in the ester groups of these quaternary compounds . . . seems to correspond . . . to that of the acid form of the omega-dimethylaminoethyl esters," it seems not unlikely that the biological activity of this tertiary amino compound could be of a similar order to that of cholinethiol itself. Thus, in the absence of 217 AO, the cholinesterase hydrolysis of the thiolester takes place and the observed responses arise from the combined actions of the ester and the thiolcholine liberated from it. In the presence of 217 AO, the inhibition of the esterase itself appears to result in the release of a cholinethiol-like compound and, due to the time which elapses before the thiolester is subsequently administered, at least part of this cholinethiol-like substance will have been converted to the corresponding disulfide leading to an even more complex situation. It is perhaps significant that the reported latency of the responses appears to depend on the nature of the cholinethiol moiety and not on the acyl grouping. Such a hypothesis awaits the test of experiment.

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