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PROTECTION AGAINST THE TOXICITY OF CHOLINESTERASE INHIBITORS BY ACETYLCHOLINE ANTAGONISTS

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Many of the effects of cholinesterase inhibitors resemble those of parasympathomimetic drugs and it is reasonable to suppose that they are due to accumulation of acetylcholine. Stewart (1952) has provided evidence that this occurs. It is therefore to be expected that animals might be partially protected against anticholinesterases if treated with ACh antagonists. Thus, atropine or scopolamine protect against di*iso*propylphosphorofluoridate (DFP) (Modell and Krop, 1946; McNamara, Koelle, and Gilman, 1946; Koster, 1946; Wescoe, Green, McNamara, and Krop, 1948), against tetraethylpyrophosphate (TEPP) (Koppanyi, Karczmar, and King, 1947; Hamburger, Freese, Cook, and Green, 1951; Douglas and Matthews, 1952; Lewis and McKeon, 1953), against parathion (Salerno and Coon, 1950; Grob, Garlick, and Harvey, 1950; Wilhelmi and Domenjoz, 1951) and against octamethylpyrophosphoramide (OMPA) (DuBois, Doull, and Coon, 1950). Curare reduces the effects of parathion poisoning in man (Grob *et al.*, 1950), and ganglion-blocking agents decrease the toxicity of DFP (Heymans, 1950) and TEPP (Hamburger *et al.*, 1951).

Anticholinesterase drugs may differ in effectiveness at the various sites where ACh acts; for example, the most striking effects of OMPA are " muscarinic " (DuBois *et al.*, 1950), whereas other anticholinesterases may also produce " nicotinic " effects such as muscular fasciculations. The work described in this paper attempts to give quantitative expression to such differential activity.

It was considered that the activity of a cholinesterase inhibitor at a particular physiological site should be related to the degree of protection exerted against its toxic effects by an antagonist of ACh specific for that site; thus, for example, the toxicity of a substance predominantly active at " muscarinic " sites should be particularly susceptible to atropine, as described for OMPA by DuBois *et al.* (1950).

It was proposed to use atropine as inhibitor of the muscarinic actions of acetylcholine, tubocurarine as inhibitor at the neuromuscular junction, and hexamethonium as inhibitor of synaptic transmission in the autonomic ganglia. By comparison of the extent to which each of these antagonists modified the toxicity of an anticholinesterase drug it was hoped to be able to express the relative activity of the latter at the three types of site.

This analysis was carried out principally upon neostigmine methylsulphate (" Prostigmin "), TEPP and two other synthetic anticholinesterases, Ro 3-0412, 3-dimethylphosphatotrimethylanilinium methylsulphate (Andrews, Atherton, Bergel, and Morrison, 1952; Burgen and Hobbiger, 1951), and Ro 3-0422, 3-diethylphosphato-1-methyl quinolinium methylsulphate (Andrews, Atherton, Bergel, and Morrison, 1954; Hobbiger, 1954).

METHODS

Two methods were used to determine the effectiveness of the antagonist in reducing the toxicity of the anticholinesterase. In the first, the animals were given doses of the antagonist already known to provide the maximum possible protection and the toxicity (LD50) of the anticholinesterase then determined. A similar procedure has been adopted in assessing protection against the toxic effects of OMPA (DuBois *et al.*, 1950), of DFP and TEPP (Hamburger *et al.*, 1951), and of TEPP (Clewe and Dreisbach, 1954).

In the second method, the LD80 of the anticholinesterase was given throughout; the dose of antagonist which reduced the mortality to 40% was then determined. This experimental design has been used by Loew and Micetich (1948) in the assessment of adrenolytic activity and by Lewis and McKeon (1953) in determining the effects of antagonists on the mortality caused by twice the LD50 of TEPP.

Albino male mice of 18-24 g. body wt. were left unfed overnight before use. Each dose was administered to a group of at least ten, but usually twenty, mice. All drugs were injected into a tail vein in a total volume of 10 ml./kg. When toxicity was determined in the

presence of one or more protecting antagonists, the drugs were mixed in a single solution, containing the required dose of each substance in 10 ml./kg. The test of each substance, or combination of substances, consisted of at least five, and usually ten or more, dose levels. Deaths always occurred within 20 min. of injection; 1 hr. was allowed before the final count. Since more than one day was usually required to accumulate sufficient results for a satisfactory dose-mortality curve, toxicity determinations in the presence of antagonists were always accompanied by the testing of one dose of anticholinesterase alone on each day, to ascertain that the mortality was of the order originally observed.

Solutions of TEPP for injection were made from a 1% solution in propylene glycol by dilution with physiological saline; all other substances were dissolved in saline. The substances Ro 3–0412 and Ro 3–0422, being very readily hydrolysed, required careful protection from moisture in the solid state.

Results

Toxicity in the Presence of Maximally Protective Doses of Antagonist

Preliminary experiments established the smallest dose of each antagonist required for maximum protection against neostigmine. Atropine sulphate reduced the toxicity to one half when used at 10 mg./kg. and no greater reduction was obtained with as much as 100 mg./kg. When given alone, 10 mg./kg. had no detectable effects, being well below the toxic range of the drug. Tubocurarine chloride gave maximum protection at 0.1 mg./kg., which is fully paralysing and just non-lethal when given alone. With doses of neostigmine less than six times the normal toxic level, mutual antagonism allows these combinations to be both non-lethal and non-paralysing. Doses of 40 mg./kg. of hexamethonium iodide were required for full protection; this level is within the toxic range of the drug, but here, again, mutual antagonism permits combinations which are non-toxic where the dose of neostigmine is insufficient to make them lethal.

The results of tests upon each of the four anticholinesterases studied are given in Table I. The figures quoted in parentheses under the heading of LD50 are confidence limits for 95% probability, calculated by the method of Litchfield and Fertig (Litchfield and Wilcoxon, 1949). The probit regression lines from which the values were obtained are shown in Fig. 1; they were fitted to the points by eye and χ^2 values for goodness of fit were calculated by the method of Fisher described by Finney (1952). The corresponding P values were 0.9 or more and none was below 0.5.

The protection afforded by the various antagonists has been calculated from the LD50's, and the method used is illustrated by the following example.

Table I

TOXICITIES OF FOUR ANTICHOLINESTERASE DRUGS, NEOSTIGMINE, TEPP, Ro 3–0412 (3-DIMETHYLPHOSPHATO-TRIMETHYLANILINIUM METHYLSULPHATE) AND Ro 3–0422 (3-DIETHYLPHOSPHATO-1-METHYLQUINOLINIUM METHYLSULPHATE), ALONE, A, AND INJECTED WITH B, ATROPINE, 10 MG./KG.; C, TUBOCURARINE, 0.1 MG./KG.; D, HEXAMETHONIUM, 40 MG./KG.; E, ATROPINE, 10 MG./KG. WITH TUBOCURARINE, 0.1 MG./KG.; F, ATROPINE, 10 MG./KG. WITH HEXAMETHONIUM, 40 MG./KG.; G, ATROPINE, 10 MG./KG. WITH TUBOCURARINE, 0.1 MG./KG. AND HEXAMETHONIUM, 40 MG./KG. (See Fig. 1)

Anticholinesterase	Test	No. of Mice Used	Slope	LD50 (mg./kg., P=0·05)
Neostigmine	A	600	7·75	0·306 (0·296 – 0·315)
	B	260	10·6	0·66 (0·636 – 0·685)
	C	70	8·9	4·19 (3·83 – 4·61)
	D	220	6·66	4·50 (4·18 – 4·83)
	E	70	9·06	8·27 (7·53 – 9·08)
	F	200	10·015	15·56 (14·8 –16·33)
	G	60	10·33	15·05 (13·9 –16·3)
TEPP ..	A	260	5·7	0·20 (0·185 – 0·216)
	B	180	14·9	0·56 (0·54 – 0·582)
	C	60	9·9	0·73 (0·673 – 0·783)
	D	180	18·35	0·666 (0·644 – 0·688)
	E	70	5·94	0·95 (0·827 – 1·09)
	F	230	12·5	0·76 (0·732 – 0·79)
	G	80	6·73	0·98 (0·875 – 1·098)
Ro 3–0412	A	300	4·17	0·497 (0·45 – 0·547)
	B	200	3·0	1·69 (1·56 – 1·83)
	C	70	10·3	7·25 (6·72 – 7·82)
	D	200	2·72	3·11 (2·92 – 3·31)
	E	60	7·85	9·25 (8·33 –10·28)
	F	200	4·34	12·5 (11·2 –14·0)
	G	50	5·66	13·5 (11·6 –15·7)
Ro 3–0422	A	340	10·2	0·024 (0·0232– 0·0249)
	B	200	28·9	0·044 (0·043 – 0·045)
	C	70	18·15	0·345 (0·305 – 0·39)
	D	200	11·6	0·10 (0·0954– 0·105)
	E	60	16·4	0·686 (0·652 – 0·722)
	F	200	5·04	1·74 (1·54 – 1·96)
	G	60	6·87	2·37 (2·08 – 2·70)

The LD50 of neostigmine, when given with atropine, is increased from 0.306 mg./kg. (test A) to 0.66 mg./kg. (test B); in this combination it thus exerts only 0.306/0.66, or 46.4% of its original toxicity. We may therefore consider that 53.6% of its toxicity is antagonized by the atropine. This becomes, generally,

$$\text{Protection } \% = 100\left(1 - \frac{\text{LD50 without antagonist}}{\text{LD50 with antagonist}}\right)$$

Values obtained in this way have been tabulated in Table II.

The figures in this table provide evidence for some degree of differentiation between the toxic actions of the anticholinesterase drugs studied. Atropine, for example, has a greater effect upon the toxicity of TEPP and of Ro 3–0412 than it has upon that of neostigmine or Ro 3–0422. Again, TEPP is the least susceptible to antagonism by both tubocurarine and hexamethonium. Against both Ro 3–0412 and Ro 3–0422, tubocurarine is a more effective antagonist than hexamethonium, and addition of atropine hardly increases the protection by tubocurarine, although it does against neostig-

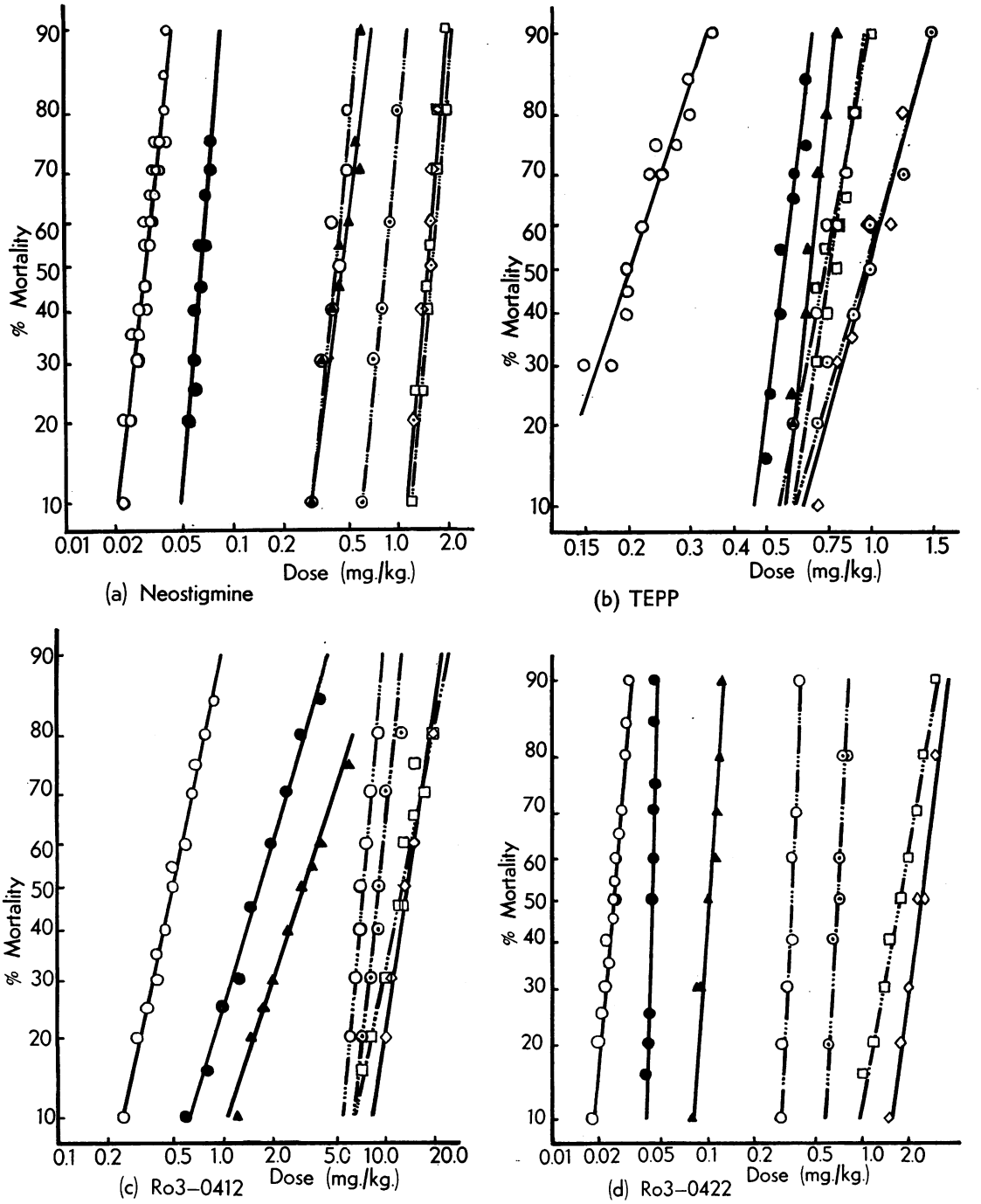

FIG. 1.—Dosage-mortality regression lines for intravenous toxicity in mice of four anticholinesterase drugs, (*a*) Neostigmine, (*b*) TEPP, (*c*) Ro 3–0412 (3-dimethylphosphato-trimethylanilinium methylsulphate) and (*d*) Ro 3–0422 (3-diethylphosphato-1-methylquinolinium methylsulphate) in the following combinations: A ○———○ Alone. B ●———● With atropine, 10 mg./kg. C ○···—···○ With tubocurarine, 0.1 mg./kg. D ▲———▲ With hexamethonium, 40 mg./kg. E ⊙···—···⊙ With atropine, 10 mg./kg. and tubocurarine, 0.1 mg./kg. F □···—···□ With atropine, 10 mg./kg., and hexamethonium, 40 mg./kg. G ◇———◇ With atropine, 10 mg./kg., tubocurarine, 0.1 mg./kg. and hexamethonium, 40 mg./kg.

TABLE II

PROTECTION OBTAINED AGAINST THE TOXICITY OF ANTICHOLINESTERASE DRUGS BY INJECTION TOGETHER WITH ATROPINE, TUBOCURARINE, AND HEXAMETHONIUM, SEPARATELY AND IN COMBINATION, CALCULATED FROM THE FIGURES IN TABLE I

Antagonists	Test (See Table I)	Protection %			
		Neostigmine	TEPP	Ro 3–0412	Ro 3–0422
Atropine	B	53·6	64·3	70·6	45·5
Tubocurarine ..	C	92·7	72·6	93·1	95·0
Hexamethonium ..	D	93·2	70·0	84·0	76·0
Atropine + tubocurarine ..	E	96·3	78·3	94·6	96·5
Atropine + hexamethonium ..	F	98·0	73·7	96·0	98·6
Atropine, tubocurarine + hexamethonium	G	98·0	79·6	96·3	99·0

mine and TEPP. In all combinations, atropine and hexamethonium give greater protection than does hexamethonium alone. Except with TEPP, addition of hexamethonium augments the protection obtained with a combination of atropine and tubocurarine. TEPP is also exceptional in that addition of tubocurarine further increases the protection by atropine plus hexamethonium. These differences are discussed more fully below.

Against TEPP, the maximum protection possible, with all three antagonists together, is about 80%, whereas against the other three anticholinesterases it is more than 95%. With neostigmine and Ro 3–0422, indeed, the protection could be as high as 98% and 99%, respectively. The greater resistance shown by TEPP toxicity could be due to the simultaneously injected antagonists having insufficient time to exert protection at sites to which TEPP might penetrate more readily. Tests were therefore performed by injecting the antagonists subcutaneously 30 min. before the intravenous injection of the anticholinesterase. Both neostigmine and TEPP were tested in this way. The results showed that the protection conferred by atropine and by hexamethonium was indistinguishable from that found with simultaneous intravenous injection.

50% *Protective Dose of Antagonist*

Fig. 2 demonstrates the derivation of the index which we have used to compare the protective effects of the antagonists against normal lethal levels of the anticholinesterases. That dose of antagonist which, injected with the LD80 of the anticholinesterase drug, reduces the mortality to 40% we have called the PD50. The relation between dose of antagonist and the mortality probit of the mixture is not strictly linear and the PD50's derived by interpolation lack the precision of the results obtained by the previous method. As each of the drugs in the mixture contributes a source of variance, too great a significance should not be attached to small differences. It has been feasible only to use each antagonist singly, and the values are given in Table III.

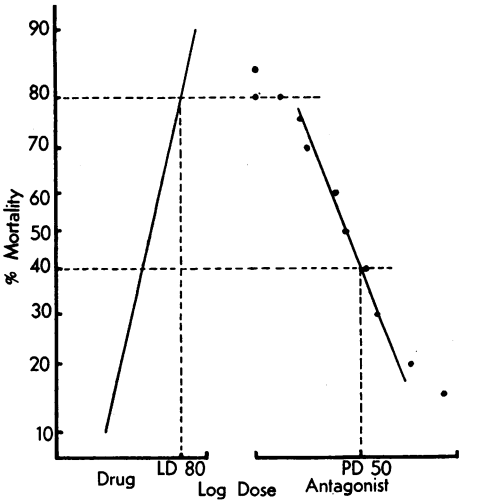

FIG. 2.—Definition of PD50 value. The regression line on the right relates mortality to dose of antagonist injected together with the LD80 of toxic drug.

TABLE III

PD50 VALUES (FIG. 2) FOR ATROPINE, TUBOCURARINE AND HEXAMETHONIUM AGAINST NEOSTIGMINE, TEPP, Ro 3–0412 AND Ro 3–0422

Anti-cholinesterase	PD50 (mg./kg.)		
	Atropine	Tubocurarine	Hexamethonium
Neostigmine ..	0·31	0·023	0·53
TEPP	0·78	0·02	1·1
Ro 3–0412 ..	3·1	0·027	5·0
Ro 3–0422 ..	2·45	0·01	5·4

It is clear that the effectiveness of an antagonist will be inversely related to the PD50. In Table III it may be seen, for example, that neostigmine is the most sensitive to antagonism by both atropine and hexamethonium, whereas Ro 3–0422 is most readily antagonized by tubocurarine. The values thus bear little relation to those obtained by the first method. This is clearly shown in Table IV, where the effectiveness of the three antagonists, as determined by each of the methods used, is expressed relative to the values obtained with neostigmine.

DISCUSSION

A drug which has several different pharmacological actions may, on administration in increasing doses, be lethal from the combined effects of these

TABLE IV

EFFECTIVENESS OF ATROPINE, TUBOCURARINE AND HEXAMETHONIUM AGAINST THE TOXICITY OF ANTICHOLINESTERASE DRUGS, RELATIVE TO THEIR EFFECTIVENESS AGAINST NEOSTIGMINE, AS MEASURED BY PD50 (TABLE III) AND BY THE VALUES FOR PROTECTION IN TABLE II

Anticholinesterase	Ratio of PD50 (Table III)			Ratio of % Protection (Table II)		
	Atropine	Tubocurarine	Hexamethonium	Atropine	Tubocurarine	Hexamethonium
Neostigmine ..	1·0	1·0	1·0	1·0	1·0	1·0
TEPP ..	0·4	1·15	0·48	1·2	0·785	0·75
Ro 3–0412	0·1	0·85	0·1	1·32	1·0	0·9
Ro 3–0422	0·125	2·3	0·1	0·85	1·025	0·815

actions. If the subject can be protected from one action of the drug, greater doses may be tolerated. If protection against each of the actions can be achieved in turn, it should be possible to obtain in this way some measure of the contribution of each to the total toxicity.

One practical difficulty is, however, that the protecting drug, or antagonist, may not be absolutely specific for any one type of site. Atropine, for instance, can interfere with the central actions of anticholinesterases (Modell and Krop, 1946; Wescoe *et al.*, 1948; Douglas and Matthews, 1952) and is capable of blocking ganglionic transmission (Feldberg and Vartiainen, 1934; Cahen and Tvede, 1953). Tubocurarine may paralyse autonomic ganglia (Brown and Feldberg, 1935; Eccles, 1943) and can exert central actions (Salama and Wright, 1950) which may themselves be modified by atropine (Salama and Wright, 1952). Hexamethonium, although predominantly active at the ganglionic synapse and with a negligible action at the myoneural junction, may also have a central action (Laurence and Stacey, 1952).

By comparing the appropriate toxicity values, according to the procedure used to obtain the values for protection given in Table II, the amount of additional protection conferred by one antagonist when given with another may be calculated. Some of the values thus obtained are presented in Fig. 3. In some combinations protective actions of the antagonist appear to reinforce each other simply; this is seen with neostigmine and Ro 3–0422 antagonized by atropine and tubocurarine; and in the antagonism of Ro 3–0412 by atropine and hexamethonium. Here each antagonist exerts the same degree of protection whether given alone or with the other. In such cases it may well be permissible to deduce the relative activities of the anticholinesterases at the site of action characteristic of each antagonist. We may conclude, for example, from Table II, that the toxicity of neostigmine derives more from effects at "muscarinic" sites than does that of Ro 3–0422, whereas effects at the neuromuscular junction are greater with Ro 3–0422 than with neostigmine.

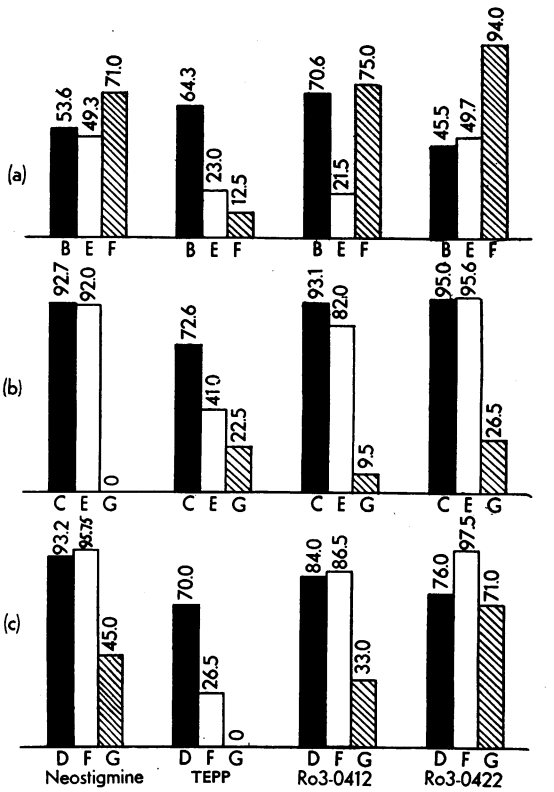

FIG. 3.—Protection obtained against the toxicity of four anticholinesterase drugs by atropine, tubocurarine and hexamethonium, alone and in combination. (Calculated from values in Table I.) (*a*) Protection due to atropine. B. Alone. (Calculated from tests B and A.) E. In the presence of tubocurarine. (Calculated from tests E and C.) F. In the presence of hexamethonium. (Calculated from tests F and D.) (*b*) Protection due to tubocurarine. C. Alone. (Calculated from tests C and A.) E. In the presence of atropine. (Calculated from tests E and B.) G. In the presence of atropine and hexamethonium. (Calculated from tests G and F.) (*c*) Protection due to hexamethonium. D. Alone. (Calculated from tests D and A.) F. In the presence of atropine. (Calculated from tests F and B.) G. In the presence of atropine and tubocurarine. (Calculated from tests G and E.)

Greater antagonism than would be accounted for by simple reinforcement is exerted by atropine and hexamethonium together against both neostigmine and Ro 3–0422; this may indicate synergism. Since the administration of all three antagonists together is no more effective against these two drugs than is the combination of atropine and hexamethonium, it may be deduced that the protective properties of tubocurarine and hexamethonium overlap to some extent. With TEPP all, and with Ro 3–0412 most,

of the combinations of antagonists afford less protection than would result from simple reinforcement, indicating overlap of effectiveness. This would seem to mean that the scope of action of an antagonist varies according to the anticholinesterase concerned; it is not clear why this should be so.

The results differentiate in several ways between neostigmine and Ro 3–0422 on the one hand and between TEPP and Ro 3–0412 on the other. TEPP and Ro 3–0412 are more susceptible to antagonism by atropine, and show a greater proportion of residual toxicity than do the other two compounds, whereas atropine and tubocurarine antagonize, without interaction, only neostigmine and Ro 3–0422. It is of interest that Hobbiger has shown (Burgen and Hobbiger, 1951; Hobbiger, 1954) that these two pairs of substances differ in the ratios of their *in vitro* activities towards true and pseudo-cholinesterase, neostigmine and Ro 3–0422 being 6 and 3.3 times as active against true as against pseudo-, respectively, whereas TEPP and Ro 3–0412 are 15 and 12 times as active against pseudo- as against true. Residual toxicity is very much greater with TEPP than with any of the other drugs. As the latter all differ from TEPP in that they are quaternary salts, it might be suspected that this points to differences in solubility, and hence in distribution. But Grob and Harvey (1949) have shown that TEPP has little lipoid solubility and so does not differ from quaternary salts in this respect.

A further point of interest concerns the nature of the toxicity remaining in the presence of all three antagonists. The ultimate cause of death appears to be respiratory failure even when, in the presence of antagonists, 100 times the normal lethal dose is necessary. De Candole, Douglas, Evans, Holmes, Spencer, Torrance, and Wilson (1953) have shown, for various organophosphorus anticholinesterases, that the contributory factors to the respiratory failure are, primarily, inhibition of the centre and, secondarily, bronchoconstriction and neuromuscular block. Douglas and Matthews (1952) found that atropine will protect against central failure and bronchoconstriction, and it might be expected that tubocurarine could relieve the depolarizing type of neuromuscular block (Dallemagne and Philippot, 1952). The mechanism, at least of the central inhibition, however, must be beyond the complete control of these antagonists, since, even in the presence of maximally protective doses, a sufficient amount of anticholinesterase can kill by respiratory failure.

The method depending on maximum protection involves giving doses of anticholinesterases many times the normal lethal levels. The distribution and mode of action of such large doses may not be the same as with small doses. It is, therefore, doubtful whether the relative activities at various sites determined by modification of the toxicity of very high doses will indicate the relative importance of these actions at normal toxic dose levels—still less at sub-lethal levels. For this reason the second method, using the LD80 of the anticholinesterase, was adopted. It requires lower doses of the antagonists and thus is likely to favour the characteristic, rather than the less typical, action of each antagonist. This method might, therefore, give a truer picture of the distribution of toxic activity of an anticholinesterase drug.

There are certain differences between the results by the two methods. Thus, of the four anticholinesterases, both methods place Ro 3–0422 as the most susceptible to tubocurarine and as relatively little, or least, affected by atropine, and neostigmine as most strongly antagonized by hexamethonium. The PD50 method shows, however, that low doses of neostigmine are more sensitive to atropine than are the higher doses used in the other method. This could indicate that the distribution or mode of action of neostigmine may differ with the dose level. Although TEPP and Ro 3–0412 are most affected by atropine by the first method, they are least affected in the PD50 method. Besides the possibility of differences in distribution and mode of action, the overlap between atropine and the other antagonists may be concerned in these differences.

Summary

1. The toxicity of four anticholinesterases—neostigmine, TEPP, and the two synthetic compounds, Ro 3–0412 and Ro 3–0422—has been analysed by comparing the effects of antagonism by atropine, tubocurarine, and hexamethonium, singly and in combination. Two methods were used. One measured the toxicity in the presence of maximally protective doses of antagonist; the other determined the amount of antagonist (PD50) required to halve the mortality of the LD80 of anticholinesterase.

2. The first method appears to differentiate between neostigmine and Ro 3–0422 on the one hand and between TEPP and Ro 3–0412 on the other. Against the first two, atropine and tubocurarine seem to act independently; combined protection by all three antagonists together is high. The second two are more strongly antagonized by atropine, but all antagonists seem to overlap in function; protection by combined antagonists is particularly low against TEPP.

3. By both methods Ro 3–0422 is most susceptible to tubocurarine and relatively little affected

by atropine, but with neostigmine the results depend upon the method used, low doses being strongly antagonized by atropine. These and other differences between the results afforded by the two methods may indicate that the distribution of the drugs, or their mode of action, varies with change in dose level.

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