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THE ANTAGONISM OF CURARIZING ACTIVITY BY PHENOLIC SUBSTANCES *

BY

G. A. MOGEY AND P. A. YOUNG

From the Wellcome Research Laboratories, Beckenham, Kent

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In the course of the biological assay of solutions of *d*-tubocurarine chloride containing *p*-chloro-*m*-cresol as a bacteriostatic agent it was found that this substance had an antagonistic action on the curarizing activity. This type of antagonism has been reported previously by Rothberger (1902), Mizuno (1933), and Coppée (1943), but has never been described for isolated mammalian tissues nor for pure *d*-tubocurarine chloride, and in view of the present-day practice of using phenolic substances as bacteriostatic agents it seemed desirable to investigate it further. Considerable confusion exists as to the identity of the so-called *p*-chloro-*m*-cresol; the *British Pharmacopoeia* (1948a) records it as 6-chloro-3-hydroxytoluene, and (*B.P.*, 1948b) quotes Wien (1939) as having established its toxicity, whereas Wien used 4-chloro-3-hydroxytoluene which is stated to be *p*-chloro-*m*-cresol in the *Merck Index* (1940). The *p*-chloro-*m*-cresol we have used is 6-chloro-3-hydroxytoluene, m.p. 63–64° (the m.p. of the 6-chloro-compound is variously given as 52–66° whereas that for the 4-chloro-compound is 46°).

METHODS

Rat diaphragm-phrenic nerve preparations as described by Bülbring (1946) have been used throughout these experiments. The capacity of the bath was 100 c.c. A fresh preparation was used for each compound. Our Ringer-Locke, aerated with oxygen, contained 0.5 g. sodium bicarbonate/litre as opposed to the usual 0.15 g./litre. Square wave impulses of maximal strength and 0.34 or 6.7 msec. duration were applied to the nerve through a fluid electrode at the rate of 5 per minute and the contraction recorded isotonically.

RESULTS

From *p*-chloro-*m*-cresol we traced the antagonism through the three isomeric cresols to phenol. Toluene was too toxic to the preparation to give any satisfactory result. Next the antagonism was demonstrated in the dihydroxybenzenes, being most potent in catechol. Of the trihydroxybenzenes, phloroglucinol was the only one in which we found the action; pyrogallol was too powerful a reducing agent and hydroxyhydroquinone was not available. Benzyl alcohol, salicylic acid, α- and β-naphthol, 2:2′-dimethoxydiphenyl ether, veratrole, and 1:3-dimethoxybenzene were inactive. In addition to antagonizing *d*-tubocurarine, catechol and phenol reduced the action on a rat diaphragm of the triethiodide of 1:2:3-tri (β-diethylaminoethoxy) benzene (R.P. 3697), of the erythrina alkaloids, β-erythroidine, and dihydro-β-erythroidine, and of strychnine ethobromide the action of which is not reversed by neostigmine. They produced only a very slight antagonism of decamethonium iodide. The addition of catechol or phenol before or after tubocurarine did not affect the result.

When it became apparent that the relative antagonistic activities of a series of phenolic compounds had to be determined, a test of standardized design was decided upon. Essentially this was to find that dose of antagonist (AD66/33) which would reduce two-thirds paralysis, produced by *d*-tubocurarine alone, to one-third. In practice, this was found by measuring the paralysis in the presence of two doses of *d*-tubocurarine, the doses being selected so as to produce, by themselves, about 80 and 50 per cent paralysis respectively. Logarithmically spaced doses of antagonist were added to each dose of *d*-tubocurarine and the paralyses displayed by the mixtures were recorded. Since the relationship between log-dose *d*-tubocurarine and percentage paralysis is known to be linear in the absence of antagonizing substances (Fig. 1), at least between 20–80 per cent paralysis (Chou, 1947; Trevan, 1948) and

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since this linearity held within the experimental limits in the presence of phenols tested at three or more concentrations of *d*-tubocurarine, the graphical method of interpolation of results shown in Fig. 2 was employed. Here, degree of paralysis is plotted against dose of *d*-tubocurarine for each level of antagonist. By drawing an ordinate through the point on the upper control lines showing 66.7 per cent paralysis the percentage for each dose of antagonist corresponding to this degree of control may be read off. These figures may then be plotted against log-dose antagonist as in Fig. 3 and the AD66/33 determined directly.

The advantages of this design of test are that it is balanced about the 50 per cent paralysis point and that, by standardizing the effect and not the dose of *d*-tubocurarine (cf. Blaschko *et al.*, 1949), the variation between rat diaphragms is eliminated.

Statistical examination of the paralysis values obtained with varying doses of antagonists in the presence of the two-thirds paralysing dose of *d*-tubocurarine has shown that for each drug these are linearly related to log dose. A χ^2 test applied to the slopes of the regression lines for eight drugs gave a value of 13.47, which is not significant at the 5 per cent probability level. Thus a common slope of 30.93 (per cent per tenfold dose increment) could be given to each substance and its AD66/33 computed. Comparison of the values so obtained was then valid. These comparisons are recorded in Table I and include activities relative to phenol, both weight for weight and on the basis of molecular weights. The fiducial limits (p 95) of the relative activities are included, and show that, while

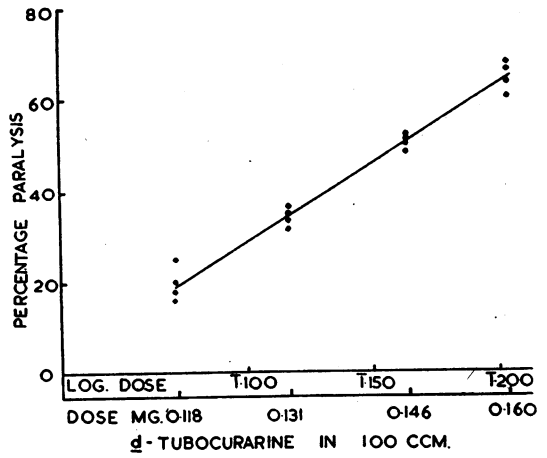

FIG. 1.—Dose-response-curve for *d*-tubocurarine on rat diaphragm showing linearity of regression. Doses (in mg.) are plotted logarithmically.

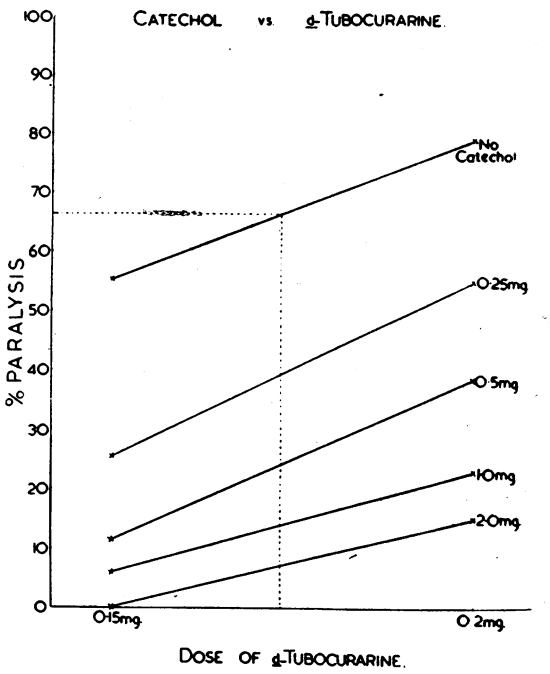

FIG. 2.—Reduction of paralysis of rat diaphragm by increasing doses of catechol added to two doses of *d*-tubocurarine. The vertical broken line is used to estimate the paralysis expected with each level of catechol in the presence of a two-thirds paralysing dose of *d*-tubocurarine.

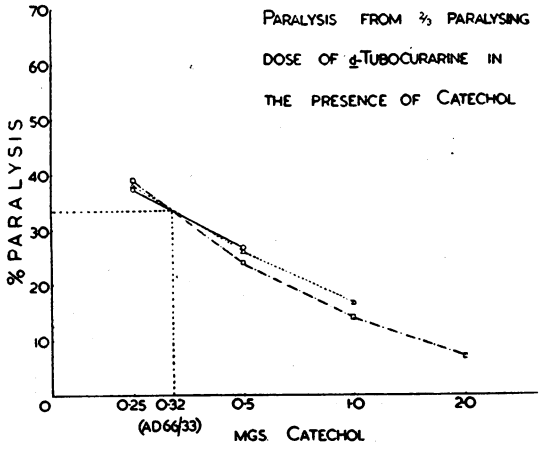

FIG. 3.—Data drawn from experiments similar to that illustrated in Fig. 2 employing 4, 3, and 2 doses of catechol. AD66/33 is the weight of catechol, read off the logarithmic scale, reducing the control two-thirds paralysis to one-third.

TABLE I

PHENOLIC ANTAGONISTS OF TUBOCURARINE CHLORIDE

Activity of antagonists estimated on rat diaphragm preparations and expressed as (1) AD66/33 or that amount of antagonist which reduces by half the activity of an amount of tubocurarine which by itself would have caused 66.7 per cent paralysis, (2) activity relative to phenol on a weight-for-weight basis, and (3) relative to phenol on a molar basis. In column (2) the p 95 limits are given in parentheses. Hydroquinone, guaiacol, and phloroglucinol were too weak to give an AD66/33.

Antagonist	(1) AD66/33 mg.	(2) Relative activity	(3) Relative molar activity
Catechol ...	0.30	5.0 (3.60–7.02)	5.9
p-Chloro-phenol	0.52	2.9 (1.89–4.56)	4.0
o-Cresol ...	0.62	2.5 (1.37–4.46)	2.8
o-Chloro-phenol	1.36	1.1 (0.78–1.61)	1.5
m-Cresol ...	1.37	1.1 (0.76–1.62)	1.3
Phenol	1.52	1.0	1.0
p-Cresol ...	1.64	0.9 (0.57–1.52)	1.1
Resorcinol ...	2.96	0.5 (0.35–0.75)	0.6
Hydroquinone	—	0.3	0.32
Guaiacol ...	—	0.1	0.13
Phloroglucinol	—	0.1	0.11

the actual relationships may not be too clearly defined, there is nevertheless a distinct fall of potency down the series. The results for hydroquinone, guaiacol, and phloroglucinol were similarly obtained by extrapolation of their graphs. The values given for these three cannot be regarded as more than an indication of their relative inactivity, however, as at high concentrations their paralysis-dose curves flattened out and with hydroquinone there was a complicating contracture of the diaphragm.

The results in Table I are essentially similar to those quoted by Coppée (1943). There does not appear to be any relationship between structure and action.

In order to investigate the absolute relationship between antagonist and *d*-tubocurarine, an alternative method of treating the information obtained from these tests was employed. The reduction in paralysis produced by the addition of antagonist to a given dose of tubocurarine may be regarded as equivalent to the removal of a portion of the tubocurarine. The mean slope of the paralysis/log dose *d*-tubocurarine regression line for the duration of the experiments was computed as being 252. If the paralysis produced by a known dose of *d*-tubocurarine be measured and its value plotted against log-dose, a line possessing the standard slope may be constructed. If, now, the paralysis be reduced by the addition of antagonist to the bath, the dose of tubocurarine which by itself would be expected to cause this smaller paralysis can be read off the graph. The difference between this dose and that in fact present may be regarded as the quantity of tubocurarine "neutralized" by the dose of antagonist. Each antagonist could then be represented by a curve relating its equivalence by weight with *d*-tubocurarine. The family of curves so obtained reflected the relative potencies already observed, in that they formed a series of parallel curves, parabolic in nature, lying in order one above another on the *d*-tubocurarine scale. The weights quoted above as AD66/33 correspond in this method to the weight of antagonist equivalent to 0.045 mg. *d*-tubocurarine, since this is the difference between the dose of tubocurarine required to produce two-thirds paralysis and that required to produce one-third paralysis.

While this method of plotting results seemed to indicate a constant activity of the different antagonists relative to one another, it was apparent that their relationship to *d*-tubocurarine was

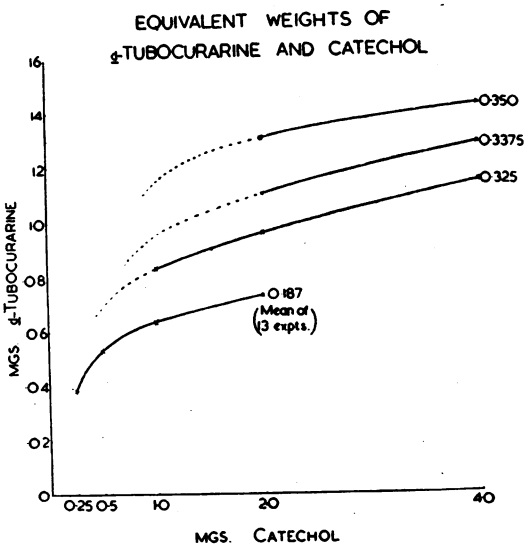

FIG. 4.—Neutralization of *d*-tubocurarine by catechol. The mean doses (mg.) of *d*-tubocurarine are indicated against the separate curves which show that the effectiveness of catechol increases as the *d*-tubocurarine concentration increases.

not stoichiometric. It appeared, moreover, that the antagonists became progressively less efficient neutralizing agents as their concentration increased. Indeed, it seemed possible that the curves were asymptotic, each substance having a limiting weight of tubocurarine which it could neutralize under our experimental conditions. However, closer examination of the accumulated data showed that the weight of tubocurarine neutralized by any one dose of antagonist increased as the actual dose of tubocurarine increased. An experiment to illustrate this point was conducted with catechol. Fig. 4 shows the mean curve for thirteen experiments in which the mean dose of *d*-tubocurarine was 0.187 mg., and three further lines obtained when catechol was added to increasing doses of tubocurarine. The latter three doses represented an excess of tubocurarine over the dose (approximately 0.275 mg.) required to produce 100 per cent paralysis.

However, when more and more excess *d*-tubocurarine is added a stage is eventually reached at which catechol will not antagonize the paralysis. This is reminiscent of the failure of neostigmine to antagonize large doses of *d*-tubocurarine (Fig. 5)

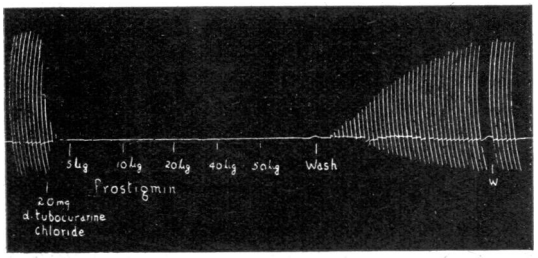

FIG. 5.—Rat diaphragm in 100 c.c. Ringer-Locke. Indirect maximal stimuli 6.7 msec. duration at 5 per min. "Prostigmin" even at high concentrations fails to antagonize a large dose of *d*-tubocurarine (5 µg. prostigmin normally reverses the action of 0.2 mg. *d*-tubocurarine.)

which Trevan (1948) has suggested may be due to *d*-tubocurarine acting at two sites, at only one of which it is reversible. Very large doses of catechol cannot be used to antagonize large excesses of *d*-tubocurarine because at high concentrations catechol has a depressant effect on the muscle fibres.

Further study of the nature of the action of catechol, as given below, showed its essential difference from the action of an anticholinesterase drug. It has been shown that a rat diaphragm, stimulated through the phrenic nerve with square wave impulses of short duration (0.34 msec.) and maximal intensity, responds with a twitch, whereas longer stimuli (6.7 msec.) produce tetanic responses (Mogey and Trevan, 1948a). Fig. 6 shows a diaphragm responding with greater and lesser contractions to long and short stimuli and also illustrates how the twitch response to short stimuli is altered to a repetitive response by eserine. This artificially induced repetitive response is abolished by minute doses of *d*-tubocurarine, doses so small that they do not affect the naturally occurring

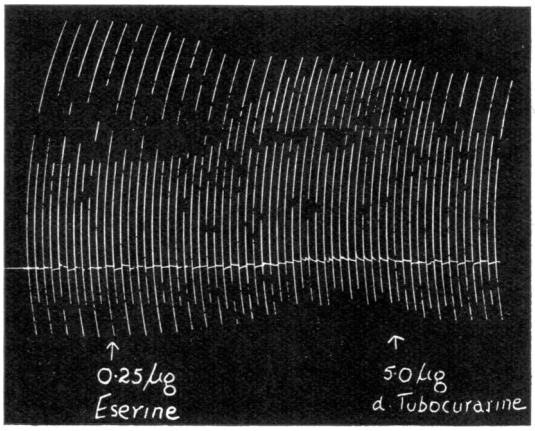

FIG. 6.—Rat diaphragm in 100 c.c. Ringer-Locke at 37° C. Alternate long (6.7 msec.) and short (0.34 msec.) stimuli of maximal intensity at 5 per min. to phrenic nerve. Eserine changes twitch to repetitive response and *d*-tubocurarine obliterates the artificially induced repetitive response at concentrations which leave unaffected the naturally occurring brief tetanus to long stimuli.

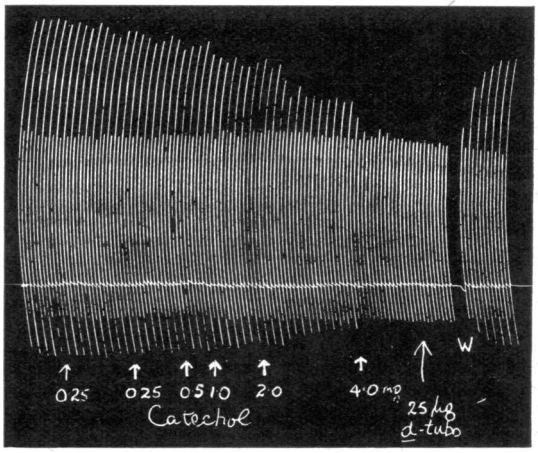

FIG. 7.—Conditions as in Fig. 6. Catechol fails to alter twitch responses while obliterating repetitive responses. *d*-Tubocurarine fails to restore repetitive responses, but they return after a wash.

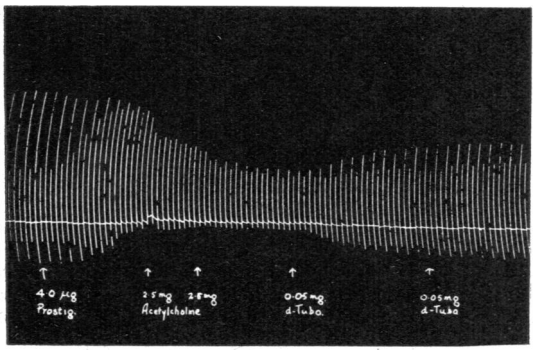

FIG. 8.—Conditions as in Figs. 6 and 7. *d*-Tubocurarine partially restores the response of the diaphragm after an acetylcholine-prostigmin paralysis.

repetitive responses to long stimuli. The ability to induce repetitive responses, common to many anticholinesterase agents, is not possessed by catechol. Fig. 7 illustrates the failure of catechol to increase the response of the diaphragm to stimuli of short duration at concentrations which increase the response during a tubocurarine-induced paralysis. Instead of inducing repetitive responses, catechol abolishes them as shown in the same figure, and, catechol having removed them, *d*-tubocurarine in low concentrations fails to restore them. This is in contrast to the action of tubocurarine on an acetylcholine paralysis (Fig. 8) (see also Bülbring, 1946—Fig. 12). Catechol failed to increase the response of the diaphragm to short stimuli of submaximal intensity and it did not improve the response of a fatigued preparation.

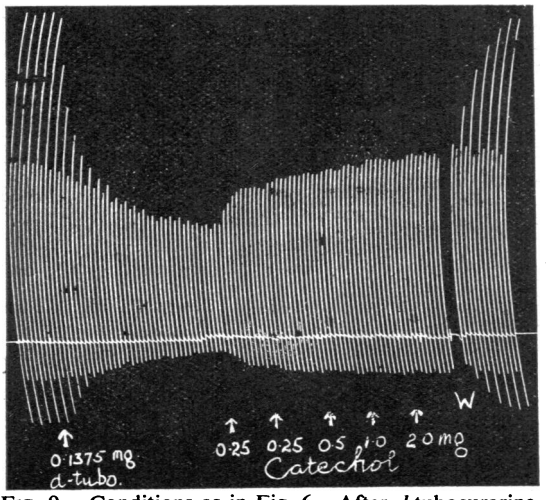

FIG. 9.—Conditions as in Fig. 6. After *d*-tubocurarine, catechol restores the twitch but fails to antagonize the action on the repetitive response to long stimuli.

The action of catechol on a tubocurarine-induced paralysis (Fig. 9) is different from that of the anticholinesterases—e.g., prostigmin as shown in Fig. 10. Its action is limited to restoration of the twitch response whereas prostigmin restores the repetitive response as well.

The anticholinesterase action of the hydroxybenzenes was examined manometrically by the method of Ammon (1933). None of those tested showed any marked inhibition of either true or pseudo cholinesterase. Results are presented in Table II.

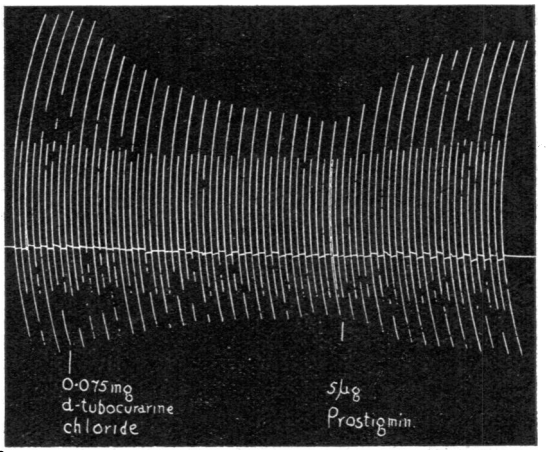

FIG. 10.—Conditions as in Fig. 6. Prostigmin, unlike catechol, restores the response to long stimuli after *d*-tubocurarine has reduced it.

TABLE II

ANTICHOLINESTERASE ACTIVITY (*in vitro*)

Concentration of inhibitor = $10^{-4}M$. Substrate for true (rat brain) cholinesterase: acetylcholine bromide. Substrate for pseudo (horse serum) cholinesterase: benzoylcholine iodide. (Each result is the mean of two determinations.)

Compound	Percentage inhibition of Rat brain enzyme	Horse serum enzyme
Phenol	4.0	1.0
Catechol	0.5	4.0
Resorcinol	8.0	0.0
Hydroquinone	9.0	0.0
Phloroglucinol	0.0	0.0
o-Cresol	1.0	1.0
m-Cresol	3.0	4.0
p-Cresol	5.0	5.0
p-Chloro-*m*-cresol ...	7.0	5.0
p-Chloro-phenol	3.0	7.0
o-Chloro-phenol	3.0	4.0
Guaiacol	0.0	0.0

The *p*H value of the Ringer-Locke, measured electrometrically, was not altered appreciably by either catechol or phenol at those concentrations which exhibited antagonism to *d*-tubocurarine.

Discussion

It is obvious that an explanation other than anticholinesterase activity must be sought for the antagonism between hydroxybenzenes and *d*-tubocurarine. Coppée (1943) has suggested that it is due to an " increase in the efficacy of the stimulus " by increasing the end plate potential (l'onde lente), but such an explanation fails to account for all the facts—e.g., catechol fails to restore repetitive responses to long stimuli after they have been removed by tubocurarine, it does not increase the response of the diaphragm to stimuli of short duration or of submaximal intensity (cf. adrenaline, Bülbring, 1946).

The antagonism cannot be due to an alteration of the action of *d*-tubocurarine by variations in H ion concentrations, for such changes are extremely slight and much too small to account for the marked antagonism. Similarly the action must reside in the hydroxybenzenes as such and not in the corresponding quinones unless it can be that the quinones, particularly *o*-benzoquinone, are highly active. Unfortunately none was available for testing.

We have not excluded a chemical combination between the antagonist and *d*-tubocurarine such as has been demonstrated for congo red and chlorazol fast pink by Kensler (1949), but such combination seems very improbable.

One other hypothesis presented itself but was subsequently proved to be false. It was based on the fact that the ratio of the potency of the dimethyl ether of *d*-tubocurarine to the potency of the phenolic alkaloid varies in different species (Mogey and Trevan, 1947; Collier *et al.*, 1948). For example, the dimethyl ether is three times as potent as the phenolic alkaloid in the rat, while it is only half as potent in the mouse. This suggested the possible importance of the hydroxyl groups in *d*-tubocurarine; in some species (e.g., the rat) a methoxyl group might have a greater anchoring power than a hydroxyl, whereas in other species (e.g., the mouse) the reverse might be true. Perhaps the hydroxyl group of the hydroxybenzenes was attaching itself to the same receptor and so relaxing the grip of the tubocurarine. If this were so then we could have expected to predict the relative potencies of anisole, guaiacol, and veratrole as antagonists of *d*-tubocurarine and its dimethyl ether in the rat and mouse. Such expectations were not confirmed; anisole was extremely toxic, causing contracture, veratrole was inactive against both *d*-tubocurarine and its dimethyl ether, and guaiacol gave results which could not be explained by this hypothesis. Catechol and guaiacol versus *d*-tubocurarine and its dimethyl ether on rat and mouse diaphragms are compared in Table III.

TABLE III

Catechol and guaiacol as antagonists of *d*-tubocurarine and its dimethyl ether on rat and mouse diaphragms.

A. RAT DIAPHRAGM

Antagonist	*d*-Tubocurarine chloride 0.2 mg.		*d*-Tubocurarine dimethylether 0.07 mg.	
	Dose of antagonist	% Paralysis	Dose of antagonist	% Paralysis
Catechol	None	75	None	79
	0.25 mg.	52	0.25 mg.	70
	0.5 mg.	36	0.5 mg.	64
	1.0 mg.	24	1.0 mg.	56
Guaiacol	None	83	None	75
	2.0 mg.	77	2.0 mg.	73
	4.0 mg.	75	4.0 mg.	73
	8.0 mg.	73	8.0 mg.	73

B. MOUSE DIAPHRAGM

Antagonist	*d*-Tubocurarine chloride 0.375 mg.		*d*-Tubocurarine dimethylether 0.7 mg.	
	Dose of antagonist	% Paralysis	Dose of antagonist	% Paralysis
Catechol	None	79	None	83
	0.25 mg.	26	0.25 mg.	12
	0.5 mg.	8	0.5 mg.	0
Guaiacol	None	91	None	89
	2.0 mg.	66	2.0 mg.	65
	4.0 mg.	54	4.0 mg.	51
	8.0 mg.	50	8.0 mg.	40

The mechanism of the antagonism is so far unexplained: it is possible that the solution lies in the almost universal attraction between phenols and proteins, thus causing steric hindrance.

SUMMARY

1. A series of hydroxy-derivatives of benzene has been shown to antagonize the action of *d*-tubocurarine on the rat diaphragm.

2. The relative potencies of the members of the series have been expressed as the concentration of antagonist which reduces by half the effect of a concentration of *d*-tubocurarine which alone would cause two-thirds paralysis.

3. The antagonism has been shown not to be due to cholinesterase inhibition or alteration of *p*H. It is suggested that it is not due to a chemical combination of antagonist with *d*-tubocurarine, but the true mechanism has not been elucidated.

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