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Interaction among Agents that Block End-plate Depolarization Competitively

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The influence of gallamine, metocurine, pancuronium, and tubocurarine on depolarization of a mammalian muscle end-plate region was examined to determine whether the antagonists given in combination exerted a degree of block consistent with the simple classical competitive model. Depolarization was produced by carbachol in isolated guinea pig lumbrical muscles and recorded by the moving fluid electrode technique. The dose–response values obtained were fitted to a regression embedded in a split plot factorial experimental design such as both to control and to measure effects of variation among preparations, order of administration, time, and level of block. Of the six possible pairings of the four drugs, four showed the simple additivity expected from a competitive mechanism, while two (pancuronium plus metocurine and metocurine plus gallamine) showed potentiation beyond additivity. In these latter two pairs the combination shifted the carbachol dose–response curve, respectively, 41 and 21% further than predicted from the classical model. The significance of this deviation in the light of alternative receptor models is discussed, and a model consistent with the observed results is outlined. (Key words: Interaction: neuromuscular relaxants. Neuromuscular relaxants: *d*-tubocurarine; gallamine; metocurine; pancuronium.)

IN AN EARLIER STUDY[1] on the effect of neuromuscular blocking agents on the indirectly elicited twitch response, we demonstrated that some combinations of so-called "competitive" neuromuscular blocking agents exhibit more than the degree of additivity to be expected on the basis of a simple competitive interaction. Specifically, the combinations metocurine plus pancuronium and gallamine plus metocurine were slightly under twofold more potent than expected, while the combinations pancuronium plus *d*-tubocurarine and metocurine plus *d*-tubocurarine were about 20% too potent. The remaining combinations examined, gallamine plus *d*-tubocurarine and gallamine plus pancuronium were simply additive. Block of the indirectly elicited twitch response can reflect both presynaptic and motor end-plate effects. In order to shed light on which might be responsible for the potentiation observed, we proceeded to the next logical step of looking at the same interactions in a system in which the nerve ending was bypassed by direct administration of an exogenous agonist.

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Methods

The experiments were carried out on isolated guinea pig lumbrical muscles bathed in Krebs' solution of the composition (mM) Na^+ 138, K^+ 5.9, Cl^- 123, Ca^{++} 2.5, Mg^{++} 1.22, dihydrogen phosphate 1.2, sulfate 1.22, bicarbonate 25, plus glucose 2.08 g/l, bubbled with 95% oxygen/5% carbon dioxide and maintained at 37° C. Depolarization produced by the bath-applied agonist carbachol was recorded by Fatt's moving fluid electrode technique,[2] as previously described.[3]

Six sets of experiments were done, one for each of the possible pairings of the four antagonists studied—gallamine, metocurine, pancuronium, and *d*-tubocurarine. All six sets were identical in format and differed simply in the two drugs paired. Each set in turn consisted of six muscle preparations. In each preparation the muscle was equilibrated *in vitro* for about half an hour, and then a control dose–response curve to carbachol was determined. For each dose the carbachol was added to the bath, depolarization was monitored, and, to minimize cumulative desensitization, the carbachol was washed out as soon as the peak response was obtained. The preparation was then left 20 min to recover, and the next dose of carbachol was given. Following the control curve, three more curves were determined in the presence of antagonist A, B and the combination "C" of A plus B (the order was determined by the statistical design; see below). Finally, the preparation was washed with antagonist-free bathing solution, and a recovery control curve was obtained to confirm reversibility. In the statistical analysis, the two bracketing control curves were combined as one. Between curves, 35 min was left for washout of the preceding agonist and for recovery of the muscle.

PHARMACOLOGICAL MODEL AND STATISTICAL ANALYSIS

The experimental design is quite complicated. The analysis parallels that of the preceding companion paper.[1]

(To avoid repetition the reader is referred to that paper for more details of the underlying rationale.)

Each preparation received three exposures to antagonist, drug A, B and the combination C. The order of administration was arranged systematically, thus

ABC BCA CAB

CBA ACB BAC

where each column contains one of the three permutations and the second row simply reverses the direction of the first. Antagonist concentration was chosen such that, on the basis of past experience, the first curve would exhibit a dose ratio of about two, the next one of three, and the last one of four. This has the disadvantage that the effect of depth of block is thereby confounded with the factor time, which was found in the previous study[1] to have a marked effect on the results. However, in the depolarization preparation we had ample experience to expect no temporal drift. We included a washout final control curve to check on this, and the results confirmed our expectations reasonably so the effect of the factor "time" (or as it turned out absence thereof) can safely be interpreted as one of level of block.

The actual calculations involved fitting theoretically determined relationships to the observed sets of depolarizations and corresponding drug concentrations and having the computer estimate the parameter values most consistent with the experimental observations along with errors of estimate. Since dose–response curves are not straight lines, curves must be fitted, so an iterative nonlinear least-squares approach was necessary. The details need not concern us here (Waud[4] gives the general rationale); suffice it to say that it involves a reasonably straightforward calculation.

The choice of curve is more central. Carbachol depolarization curves are the usual sigmoidal shape. Figure 1, which illustrates some actual experimental results, indicates that we tended to stay in the lower half of the curve (to minimize desensitization) but that at the higher levels of response the curve is starting to level off. In all six assays this bend was enough to allow the computer to fit the curves with a function of the form

$$y = H_i \cdot x^S/(x^S + K_i^S) \qquad (1)$$

where y is depolarization, x is concentration of carbachol, while H_i determines the maximum, K_i the ED_{50}, and S the steepness; i labels the preparation (see below). Note that the function (1) is completely empiric; it is chosen simply because it fits sigmoid curves well and it is easier to handle mathematically than the other standard, the probit.

Given the nature of the measurement of depolarization (depending as it does, for example, on the degree of electrical shorting, which can vary from preparation to preparation) the basic ED_{50}, K, would be expected to vary from muscle to muscle. Therefore, we used a separate K for each preparation; this is indicated by the subscript i, which labels the preparation. Similarly, six values of H were used in the actual calculations.

Now we must superimpose the classical competitive model to handle the curves in the presence of antagonist(s). We are dealing with the system

$$D + R \rightleftarrows DR \rightarrow \text{depolarization}$$

$$A + R \rightleftarrows AR$$

$$B + R \rightleftarrows BR$$

where D represents the depolarizing agent (agonist), R the receptor, and A and B two competitive antagonists. K_D, K_A, and K_B are the corresponding drug-receptor dissociation constants. It is easily shown that the effect of the antagonist(s) is simply to make the depolarizing agent look less potent by a factor

$$1 + A/K_A + B/K_B \qquad (2)$$

This means that, in the presence of antagonist, our original K_i in equation (1) is modified to become

$$K_i \cdot (1 + A/K_A + B/K_B) \qquad (3)$$

This expression, in fact, applies to all four curves. When A and B are both zero we have the control curve. When one or the other is zero we have curves A and B. When neither is zero we have the combination curve.

Finally, we add parameters to incorporate the factors "permutation," "direction," "time" (= intensity of block), and "combination." The details of this are spelled out in the companion paper[1]; the end result is that the expression in (3) is multiplied by one or more of the parameters P, D, T, and C. For each set of six preparations dealing with a specific pair of antagonists, the computer fits a series of variants on the basic function of equation (1) and, from the goodness of the fit, yields estimates of the parameters along with their standard errors and then does an analysis of variance that tells us whether the contribution of the various parameters was significant or not. We may illustrate briefly with C, the factor of prime interest. If antagonists A and B are simply additive, then when they are added in combination, the curve should be in line with the expression (3). However, we can give the computer an extra parameter C to be used to bring the combination

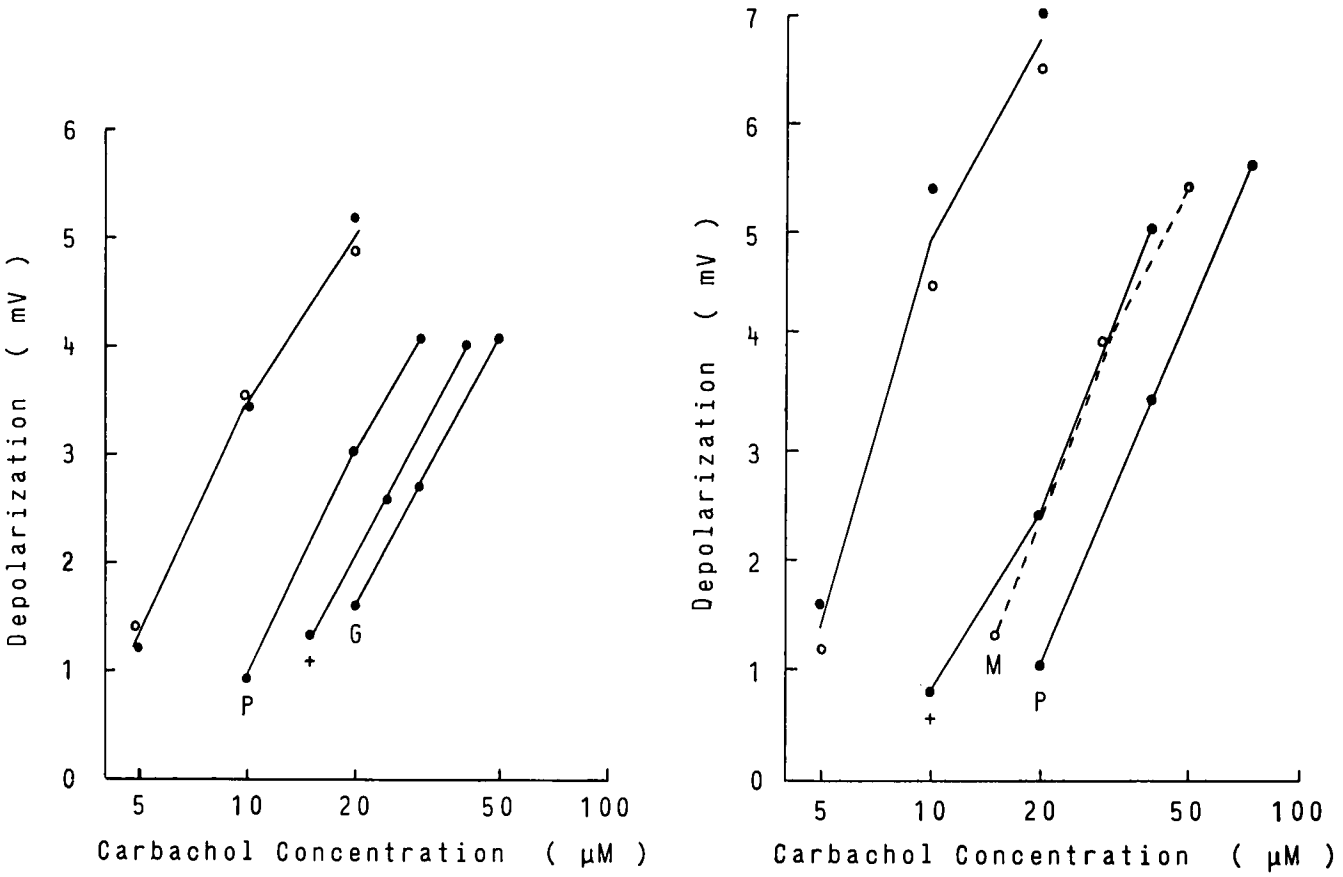


FIG. 1. Two sample experiments. Depolarization *versus* concentration curves for carbachol in the presence of antagonist A, antagonist B, and a mixture. Ordinates: depolarizations (mV). Abscissae: concentrations of carbachol (micromolar). Left hand curves: no antagonist (closed circles before, open circles after the remaining three curves). The left hand panel shows an example with no sign of potentiation. The curve P was in the presence of a concentration (0.025 μM) of pancuronium expected to give a dose ratio of about 2. The curve + was in the presence of a mixture of 0.025 μM pancuronium with 0.5 μM gallamine, concentrations such that a dose ratio of 3 was anticipated. The curve G was in the presence of gallamine 1.5 μM, a concentration expected to give a dose ratio of about 4. The right-hand panel gives an example showing potentiation. The curve + was obtained in the presence of concentrations of metocurine and pancuronium, 0.025 μM and 0.0125 μM, respectively, expected to give a dose ratio of 2. The curves M (0.1 μM metocurine) and P (0.075 μM pancuronium) were designed to give dose ratios of about 3 and 4. The curve + lies further to the right than expected.

curve still closer to the observed values. We then look at the improvement in fit. If it is statistically trivial, we conclude C added nothing, and there is no sign that the drugs do other than add as expected from simple competition. If, on the other hand, use of C leads to a significant reduction in the "error sum of squares" (the statistical measure of scatter of the observed points about the fitted lines), then we must conclude the drugs are not simply additive. Furthermore, the computer will give a numeric estimate of C. If C, for example, were 1.2, then we would conclude the antagonists were 20% more potent in combination than would have been expected from their individual effects.

Graphing the results is somewhat complicated. The control curve and the curves in the presence of A and B alone would present no problem, and that in the presence of the combination of antagonists does, because the equivalent of an ED_{50} becomes a two-dimensional entity. We have approached this problem in a manner analogous to that used earlier[1]; we have normalized all concentrations. Thus, we have generated figures 2 through 7 by drawing first a reference curve with an ED_{50} of unity and a slope governed by the fitted values of steepness parameter S. Then, for all the points, we calculate a normalized concentration by dividing the actual concentration by the expression (3) multiplied by a factor that reflects whether the curve was first, middle, or last, but without the C parameter. If the observed depolarizations, normalized by dividing by the appropriate H_i for that preparation, are then plotted against the normalized concentrations, the points should be scattered randomly about the reference curve if the

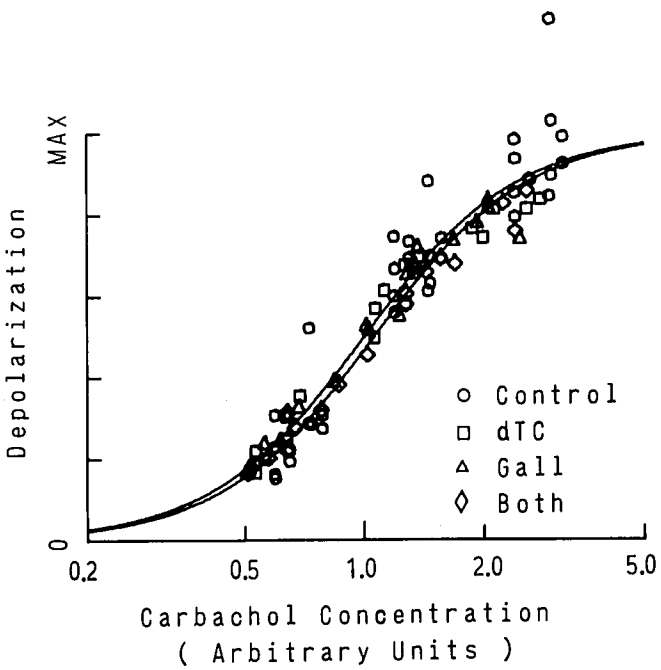


FIG. 2. "Interaction plot" for the gallamine plus *d*-tubocurarine pair. Ordinates: Depolarizations as a fraction of the maximum associated with each preparation. Abscissae: normalized carbachol concentrations. The reference curve (the left-hand one in the figure) is drawn with an ED_{50} of 1 and the steepness parameter, 2.25, derived from the least-squares fit. Circles, squares, and triangles represent observed responses in the absence of antagonist, in the presence of tubocurarine and in the presence of gallamine, respectively. In all cases the observed depolarization was divided by the maximum value of the least-squares curve fitted to the relevant preparation and the micromolar concentration was normalized by dividing by $K_i(1 + A/K_A + B/K_B)T_j$ (see text) to give values that should have an average ED_{50} of 1 and therefore lie over the reference curve. The values for the A and B combination (diamonds) are plotted analogously but will lie over the reference curve (as happens in this case) if the antagonists are simply additive. The right-hand curve was drawn parallel to the reference curve and shifted to the right by the value (1.06, see table 1) estimated for the combination factor C.

drugs A and B just add competitively. However, if there is potentiation the points for the combination curve will lie systematically to the right of the reference by a factor equal to the C parameter. To aid visualization of this last set of points (diamonds in figs. 2–7) we have added a second curve, parallel to the original reference curve (*i.e.*, same S) but shifted to the right by a factor C. Effects demonstrable at the 95% probability level were considered "significant."

Results

Figure 1 gives examples of the results of two muscles. The dose–response curves "shift to the right" in a reasonably "parallel" fashion as expected. Generally there is little sign that the curves are starting to flatten

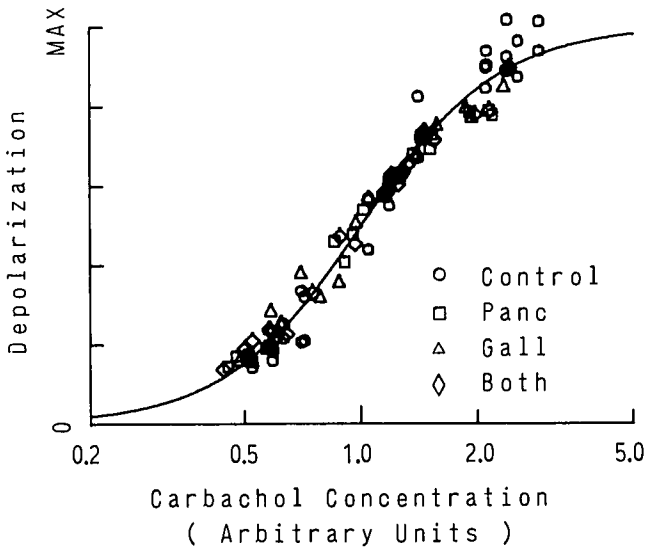


FIG. 3. "Interaction plot" for the gallamine plus pancuronium pair. Format as in figure 2.

as they approach the maximum; in other words, the responses have been kept reasonably low (to avoid desensitizing the preparation). The final three control points (open circles) are consistent with the earlier values; the sensitivity of the preparation is stable. In the left hand panel of figure 1 the three right hand curves represent response to carbachol in the presence of concentrations of pancuronium (P), gallamine (G), and a mixture (+) chosen to give dose ratios of 2, 4, and 3, respectively. The location of the curves is reasonably in line with expectation. The right-hand panel provides a

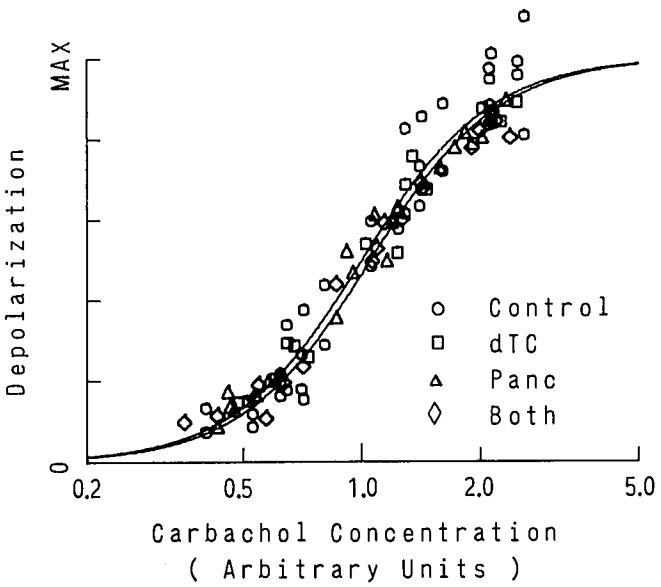


FIG. 4. "Interaction plot" for the pancuronium plus *d*-tubocurarine pair. Format as in figure 2.

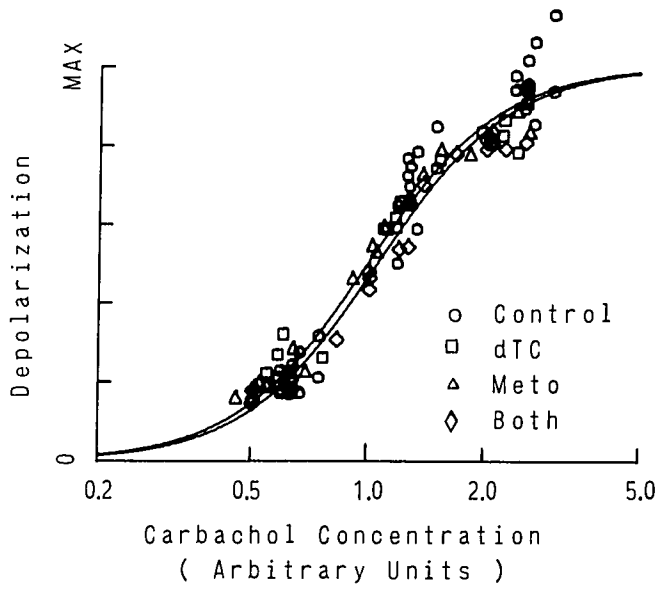


FIG. 5. "Interaction plot" for the metocurine plus *d*-tubocurarine pair. Format as in figure 2.

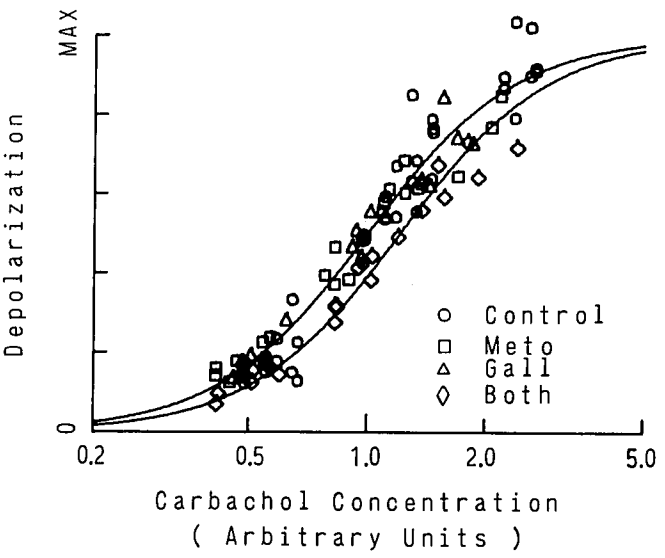


FIG. 7. "Interaction plot" for the metocurine plus gallamine pair. The degree of potentiation is less than in figure 6 but still significant.

contrast. Here the three curves for metocurine (M), pancuronium (P), and the mixture (+) should lie at dose ratios of 3, 4, and 2, respectively. Those for M and P do so, but that for the mixture lies too far to the right, *i.e.*, the dose ratio is too big when the drugs are mixed.

Figures 2–7 summarize all the basic results pictorially in "interaction plots." For the first four pairs of antagonists the combination points (diamonds) are scattered evenly over the other two sets and all lie over the line with unit ED_{50}. Furthermore, the second line is displaced only a small distance from the reference line, compared with the background scatter. All these features indicate no evidence for a deviation from simple additivity, and the analysis of variance confirmed this objectively with a nonsignificant variance ratio. For the last two pairs the picture is different. In figure 6 the combination points are clearly distinguishable from the rest. In figure 7 a shift seems likely. The corresponding analyses of variance confirmed that both differences were significant at the 99% level and gave estimates of C of 1.41 (±0.058 SE) and 1.21 (±0.050 SE) for the pancuronium plus metocurine pair (fig. 6) and gallamine plus metocurine pair (fig. 7), respectively. Table 1 summarizes all the estimates of C.

In the analysis of variance designed to detect an influence of "permutation" (order of curves ABC *vs.* BCA, for example) or "direction" (CBA *vs.* ABC) no significant effects were detected. The "time" factor,

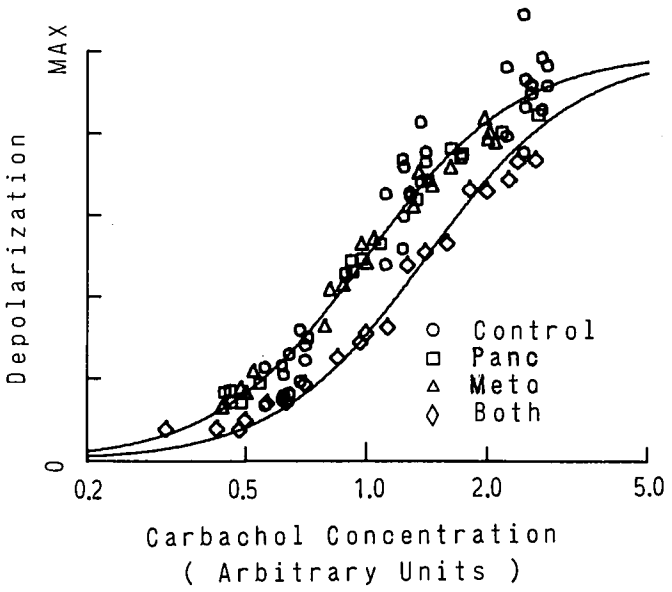


FIG. 6. "Interaction plot" for the metocurine plus pancuronium pair. With this combination, there is significant potentiation; the diamonds lie clearly to the right of the other points.

TABLE 1. Summary of Estimates of the Potentiation Factor C

Interaction	C*	SE (C)
Gallamine plus *d*-tubocurarine	1.06	0.054
Gallamine plus pancuronium	1.00	0.029
Pancuronium plus *d*-tubocurarine	1.05	0.043
Metocurine plus *d*-tubocurarine	1.06	0.037
Metocurine plus pancuronium	1.41†	0.058
Gallamine plus metocurine	1.21†	0.050

* The parameter C is unity for simple addition, greater than one if the interaction is more than additive.

† Significantly different from unity at the 99% probability level.

which would also reflect an influence of magnitude of block, showed no consistent pattern. Of the six assays, three gave a nonsignificant effect of time, one was significant at the 95% probability level, and two were significant at the 99% level.

Over the six assays the steepness parameter S gave an average value of 2.42 with a standard deviation of 0.16.

Discussion

The experimental design has enabled us to achieve precision sufficient to detect significant deviations from simple competitive kinetics. The estimates of the degree of potentiation have coefficients of variation averaging 4% (±0.7% SE). The scatter of points about the fitted curves in figures 2–7 is quite respectable, with the possible exception of an occasional high point from the original control curve. This presumably reflects the propensity of large responses to depress subsequent sensitivity. It was to avoid such desensitization that we tried to stay generally at lower response levels. The fact that an occasional point was perhaps slightly higher than this comes about because we wanted to use responses that were as high as possible, short of producing a lot of desensitization so that the steeper part of the dose–response curve would contribute to and therefore increase the sensitivity of the assay. This compromise seems to have been balanced acceptably.

The occasional significant effect of time presumably reflects this same phenomenon. If a point at the top of the early curve were high, then the subsequent curves may not be able to match this level because of desensitization and the "time" factor might pick this up. There was no marked trend with time; the three time parameters T_j averaged 1, 0.966, and 0.936 (SE 0.034). This contrasts with the early study[1] on twitch responses where one would expect, and indeed we found, a marked steady temporal drift.

The steepness of the curves not only influences the sensitivity of the assay[5] but also has pharmacologic significance. Our parameter S corresponds to a Hill coefficient of the order of two, and thus the observed values imply cooperativity or other than 1:1 stoichiometry (we return to this theme later).

We were pleased that the algorithm was generally able to estimate the maxima of the dose–response curves, even though there was often only a barely perceptible hint that they were beginning to top. Originally we had anticipated trouble and included an option in the program to fix the maximum at some reasonable value determined by the operator. This would have the disadvantage of possibly injecting subjective bias. The extent of such a problem would probably not have been great; the fitted values are close to where we would have estimated them. Nevertheless, it is more satisfying to have no subjective element introduced. Finally, we should point out that the key issue—whether there is potentiation or not—is quite insensitive to the actual value chosen for the maximum. Thus, although the concentrations used were barely high enough to estimate the maximum, and therefore that estimate is rough, this has negligible bearing on the main objective of the experiments, and the lack of precision in estimation of the relatively unimportant maxima was presumably offset by the reduced effect of desensitization achieved by avoiding large depolarizations.

The reader should note a potential source of confusion. In the companion paper[1] the potentiation factor entered into the equations in such a fashion that potentiation was associated with a value less than unity. In the present study the reverse is true; potentiation is implied by a value greater than one. The reason for this was an attempt to conform to the way one usually thinks about the two respective dose–response curves. With the twitch response of a nerve–muscle preparation, potentiation would be identified as a shift of the curve to the left, *i.e.*, a lower ED_{50}. Thus, we chose an index that would be smaller with potentiation. On the other hand, with a set of depolarization curves the effect of potentiation would be a heightened "shift to the right." Hence, we set up the model so the parameter C would be greater than one if potentiation occurred.

The precision of the estimate of the potentiation factor is slightly lower than in the earlier study.[1] (4% coefficient of variation here *vs.* 3% earlier). The higher precision of the earlier assay presumably reflects the steeper curve one obtains with twitch responses, since generally sensitivity of an assay is governed by the slope of the dose–response curve.[5]

The present results are particularly interesting in comparison with those[1] obtained earlier. In both studies the pairs gallamine plus *d*-tubocurarine and gallamine plus pancuronium showed no signs of potentiation. In both, the metocurine plus pancuronium and the gallamine plus metocurine pairs showed significant effects. Finally, with the remaining pairs, pancuronium plus *d*-tubocurarine and metocurine plus *d*-tubocurarine, there was a slight potentiation seen when the twitch response was examined, but this did not carry over to the depolarization assay. Overall these comparisons suggest, first, no bizarre behavior such as a marked effect at the end-plate not reflected in transmission. Next, one must consider whether effects on the presynaptic terminal might be involved. The most direct interpretation is simply that there is no evidence for a presynaptic

contribution. In particular, it should be noted that the concentration of antagonist needed to block the twitch is, because of the magnitude of the margin of safety of neuromuscular transmission, larger than that required to produce the dose-ratios of 2, 3, and 4 in the depolarization curves. Consequently, it is likely that the greater apparent ability of the twitch experiments to demonstrate the potentiation simply reflects a generally higher concentration of antagonist and hence a greater propensity to show the phenomenon. In any case one can make a reasonable argument that there is no pressing need to invoke a presynaptic contribution to the potentiation. This, combined with the principle of economy of hypotheses would focus us on the end-plate for an explanation. However, although there is not direct evidence of a presynaptic effect one might argue weakly *post hoc* that, although there is clearly a postsynaptic element, there might also be a secondary contribution from the nerve terminal as well.

This brings us to a very significant question. Given that there is real potentiation and that it is largely, if not completely, postsynaptic in origin, it is of interest to seek the underlying basis. Clearly the simplest classical model must be abandoned or modified. There are two particular alternatives or variants that exist as precedents: channel blocking and receptor subunit asymmetry. We consider these in turn.

Channel Blocking

It is becoming increasingly clear that positively charged quaternary compounds that react with the negatively charged receptor to the transmitter may also drift into and plug the associated membrane channel.[6] This phenomenon has been seen with tubocurarine[7,8] as well as gallamine.[9] Rang[10] gives a recent summary. Kinetically the antagonist behaves as if it binds to the open form of the receptor–pore complex rather than the closed form. Thus, agonist alone is represented by the scheme‡

$$A + R \underset{K_A}{\rightleftarrows} AR \underset{\alpha}{\overset{\beta}{\rightleftarrows}} AR^*$$

in which once drug reacts with the receptor there is rearrangement in which the channel opens to form the activated form AR*. The parameters β and α are the rate constants for channel opening and closing.[7] For two competitive antagonists B and B′, the scheme expands to

$$\begin{array}{ccccc} A + R & \overset{K_A}{\rightleftarrows} & AR & \underset{\alpha}{\overset{\beta}{\rightleftarrows}} & AR^* \\ & & \Big\Vert\downarrow K_B \text{ (and/or } K_{B'}) & & \\ & & BR \text{ (and/or } B'R) & & \end{array}$$

while channel blocking by the same drugs would be pictured by

$$\begin{array}{ccccc} A + R & \overset{K_A}{\rightleftarrows} & AR & \underset{\alpha}{\overset{\beta}{\rightleftarrows}} & AR^* \\ & & & & \Big\Vert\downarrow K_B^* \text{ (and/or } K_{B'}^*) \\ & & & & AR^*B \text{ (and/or } AR^*B') \end{array}$$

From these schemes one can derive expressions for the behavior of dose–response curves to get

$$\frac{AR^*}{Rt} = \frac{\alpha}{\alpha' + \beta} \cdot \frac{A}{A + \beta K_A/(\alpha' + \beta)} \qquad (4)$$

and

$$\alpha' = \alpha(1 + B/K_B^* + B'/K_{B'}^*) \qquad (5)$$

for the case of pure channel block by two antagonists B and B′. The case of mixed competitive and channel block leads to an even more complicated expression, so we shall confine our comments to the simpler case, which, fortunately, suffices to make the point. The control curve is given by equation (4) with $\alpha' = \alpha$, i.e. with B and B′ zero in equation (5). Thus, the effect of the antagonist will be to change α', which, from inspection of equation (4) will imply not only a change in the effective K_A but also a decrease in the maximum depolarization. Furthermore, if the curve has shifted fourfold (as in one-third of our curves), the maximum should come down markedly, since the maximum is determined by

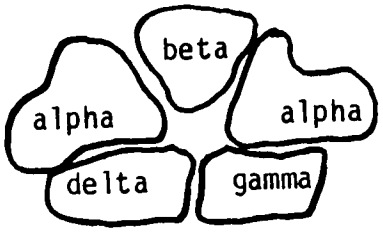
$$\frac{1}{1 + B/K_B^* + B'/K_{B'}^* + \beta/\alpha}$$

The ratio β/α is about 0.5 (compare figs. 1 and 5 of the analysis by Magleby and Stevens[11]), so at a dose ratio of 4, where $B/K_B^* + B'/K_{B'}^*$ should be about 3, the maximum should fall from 1/1.5 = 0.67 to 1/4.5 = 0.22 or to 14% of its control value. Clearly, no shift anywhere near this size was seen, so one must conclude channel blockade is not producing a perceptible effect and therefore does not look like a plausible basis for any explanation of the potentiation.

‡ So far we have used A for one antagonist, B for the other, and C for the combination. This convention led to ABC for the three curves, a convenient representation to remember. However, we are now at a point where we must consider rather complex conceptual models. Here it will prove less confusing if we use A for agonist and B for antagonist. A second antagonist then can be represented by B′. The A *versus* B distinction will now help one keep agonist *versus* antagonist in perspective. It will also bring the symbols closer in line with those in the related literature.

Monomer Asymmetry

Here we focus on a model developed elegantly by Palmer Taylor and his colleagues (to avoid a dilation of our list of references we refer to the reader to a key paper[12] and a brief review[13] which will serve as a convenient entry to the literature if more details are sought). One can start with a deductive argument from the nature[14,§] of the receptor channel complex. This entity is composed of five protein subunits, two of which have the same amino acid sequence. The five monomers are arranged pentagonally in an array which might be represented



with a central channel. Now two features are particularly relevant to our present interest. First, it is the alpha subunits which bind the transmitter. This means we have two binding sites per receptor-channel unit. Secondly, although these two units have the same amino acid sequence, they might be expected to differ functionally since they are in different environments (i.e., one would be bordered by a beta and a gamma, the other by a beta and a delta). Taken together these two features suggest that (i) one might have to activate two subunits to open the channel and (ii) drugs might bind to the two alpha units with different affinities. Taylor and his group have found that, indeed, this seems to be the case.

We turn now to the implications for our two studies. (We include the results of the previous study[1] on the response to an indirectly elicited twitch both because any explanation should apply to those observations as well and because those results seem to have picked up levels of non-additivity which could not be detected in the depolarization assay.)

Unfortunately the argument becomes rather complicated at this point because we have to consider three drugs reacting with two different receptor types. We have therefore placed the more mathematic details in an appendix and will focus here on the results. In essence what we have done is to calculate what values we should expect for the magnitude of the interaction parameter C. The results are gathered in table 2 (for the earlier studies on the twitch response) and table 3 (for the present studies on the depolarization response). (To facilitate comparisons between the two sets of numbers, we have used the reciprocal of the interaction parameters from the earlier study, so that a value greater than unity implies more than additivity throughout both tables 2 and 3.)

The tables require explanation. The key feature that had to be introduced was the splitting of a single dissociation constant K_B for the B + R reaction into two, K_B and $\underline{K}_B$ for the B + R and B + $\underline{R}$ reactions, where absence or presence of the underline labels one or the other of the two alpha subunits. Similarly we have a $K_{B'}$ for the second antagonist B'. The classical model now represents the special case where $\underline{K}_B = K_B$ and $\underline{K}_{B'} = K_{B'}$. This indicates that we can view the extent of alpha unit asymmetry in terms of the extent to which the ratios $\underline{K}_B/K_B$ and $\underline{K}_{B'}/K_{B'}$ differ from unity. Tables 2 and 3 have been set up this way. We varied $\underline{K}_B/K_B$ and $\underline{K}_{B'}/K_{B'}$ widely (Taylor's studies gave us a rough idea of how far to range; he reported ratios between 4 and 89.[13] Since these could represent $\underline{K}_B/K_B$ or $K_B/\underline{K}_B$, we focused on the range 0.01 to 100) and calculated expected values of the interaction parameter. We then compared these values with those we observed and, for each of the four drugs studied, sought an assignment of K_B ratios that might give a consistent picture overall. We finally extracted from the original large array those sections close to our assigned values and summarized them in Parts A of tables 2 and 3. To illustrate we may walk through part A of table 2. The columns represent ratios of $\underline{K}_B/K_B$ for one drug, B. The rows represent that ratio for the second drug, B'. The main entries in the table are the calculated values for the interaction parameters given the values of $\underline{K}_B/K_B$ and $\underline{K}_{B'}/K_{B'}$ indicated by the upper and left margins. These theoretic values are not italicized and are given to three decimal places, not for precision but simply to help distinguish them from the observed values. The latter are italicized and given to only two decimal places. Now let us consider a specific interaction, that between gallamine (G) and pancuronium (P). On the basis of a trial-and-error process, but with some guidance from Taylor's results (see below), we have assigned a ratio of $\underline{K}_B/K_B$ between 5 and 10 to gallamine and one between 10 and 20 to pancuronium. These assignments, and those for metocurine (M), and tubocurarine (T), are indicated by the location of the symbols G, M, P, and T along the lower and right-hand borders. The value of *0.96* at the intersection of the G row and P column represents what we found experimentally. The values 0.975, 0.988, 0.954, and 0.948 surrounding the *0.96* are the calculated values from our original master table for those cells

§ A less technical review is available in Neuroscience Commentaries 1:124–138, 1983.

TABLE 2. Values of Interaction Parameters (Twitch Response)

		K_B/K_B					
		1	5	10	20		
Part A							
	0.02	1.253	1.617	1.807	2.007		
		1.18	*1.75*	*1.83*		M	
	0.05	1.164	1.459	1.617	1.785		
$\frac{K_{B'}}{K_{B'}}$	1		1.051	1.102	1.164	T	
	5		*1.07*	0.975	*1.20*	0.988	G
	10			0.954	*0.96*	0.948	P
	20						
		T	G	P			
		K_B/K_B					
		1	5	10	20		
Part B							
	0.01	1.319	1.727	1.937	2.159		
		1.18	*1.83*	*1.75*		M	
	0.02	1.253	1.617	1.807	2.007		
$\frac{K_{B'}}{K_{B'}}$	1		1.051	1.102	*1.07*	1.164	T
	5		*1.20*	0.975	*0.96*	0.988	P
	10			0.954		0.948	G
	20						
		T	P	G			

Values in table represent magnitudes of interaction parameter for various values of K_B/K_B (columns) and $K_{B'}/K_{B'}$ (rows) where B and B′ are the two antagonists being compared. Values not underlined are calculated from the dimer receptor model with $A/K_A = 0.01$ and a dose ratio of 10. Values italicized are observed values from experiments assaying block of indirect twitch response.[1] Calculated values were obtained on a 1-2-5-10 grid. In part A, antagonists were placed in that grid (as indicated by the labels on the right and lower margins) to give reasonable agreement between calculated and observed numbers. In part B, antagonists G, M, and P were placed on the basis of assayed potency ratios in a mouse cell line[12] and T (not examined in that study) placed as in part A. To reduce clutter, only those calculated numbers bracketing observed values are retained in the table.

TABLE 3. Values of Interaction Parameters (Depolarization Response)

		K_B/K_B					
		1	5	10	20		
Part A							
	0.02	1.055	1.192	1.248	1.296		
		1.06	*1.21*	1.41		M	
	0.05	1.042	1.163	1.213	1.256		
$\frac{K_{B'}}{K_{B'}}$	1		1.016	*1.06*	1.029	*1.05*	1.042	T
	5			0.960	*1.00*	0.955	G
	10			0.943		0.933	P
	20						
		T	G	P			
		K_B/K_B					
		1	5	10	20		
Part B							
	0.01	1.061	1.205	1.264	1.314		
		1.06	*1.41*	1.21		M	
	0.02	1.055	1.192	1.248	1.296		
$\frac{K_{B'}}{K_{B'}}$	1		1.016	*1.05*	1.029	*1.06*	1.042	T
	5			0.960	*1.00*	0.955	P
	10			0.943		0.933	G
	20						
		T	P	G			

Table format as for table 2. The experimental values (italicized) are from the present end-plate depolarization studies. The calculated values were based on a value of A/K_A of 0.01 and a dose ratio of 3 (chosen to be in the middle of the values 2, 3, and 4 in the actual experiments). The assignments of position for the four blocking agents are taken from table 2 to show that the same assignments give comparably respectable fits to the depolarization data.

closest to the region of interest (*i.e.*, values of $\underline{K}_B/K_B$ of 10 and 20 and values of $\underline{K}_{B'}/K_{B'}$ of 5 and 10). Note that the calculated values bracket the observed value remarkably well. Similarly, when one looks at the rest of the table, the observed values fall very satisfactorily in line with the neighboring calculated values. Part A of table 3 gives corresponding values for the depolarization experiments of the present article. However, this time we have kept the same assignments as in table 2 because we were particularly interested to see if our model would predict results in both studies in a consistent fashion. Inspection of the table indicates this is indeed the case.

We have included a second set of assignments in Parts B of the two tables. Here we have placed G, M, and P on the basis of the observed ratios (14.8, 89.0, and 7.6, respectively) reported in Taylor's studies.[12] Since they did not give a value for T, we assigned it as in Part A. Despite the additional constraint introduced by this approach, the fit is still very respectable. The main difference between Parts A and B is a somewhat smaller $\underline{K}_{B'}/K_{B'}$ ratio for M and a reversal of the ranking of G and P. In fact, one might hardly expect too close an agreement. Taylor's studies were done in mutated cells from mice, ours in normal cells from guinea pigs.

Thus, the overall impression from tables 2 and 3 is one of excellent agreement between theory and observation.

One other aspect of the implication of two receptor units is of interest. If the agonist must react with both sites to open the channel then one would expect the reaction to be second order in A. This should be reflected in the slope of the dose–response curve by a Hill coefficient of 2 rather than the 1 associated with the simpler one-to-one first-order reaction. The slope parameter S we used to fit our curves (*c.f.*, equation [1]) is equivalent to a Hill coefficient. Thus, it is particularly interesting to note that our dose–response curves were fitted, on the average, by a value of 2.42 (SE of ±0.07). This is much more readily reconciled with second-order than first-order kinetics.

One other corollary of the proposed model deserves mention. We now have an explanation for the depolarization experiments not picking up some supraadditive interactions found in the earlier[1] study. The calculations in tables 2 and 3 indicate quite clearly that the deviation from unity depends on the magnitude of the antagonist effect, *i.e.*, the predicted numbers in table 2 are all bigger than their corresponding values in table 3. The tables differ only in that table 2 was keyed to the dose ratio, ten, appropriate[15] to blocking a twitch response, while those in table 3 to a value, three, similar to that used in the depolarization studies. (One might argue after the fact that the depolarization studies should have been done at a higher level of dose ratio. However this is not practical.) The larger concentrations of drugs that would be involved would probably raise more problems (such as desensitization or channel plugging than would be solved).

Note that our experiments are kinetic experiments, *i.e.*, descriptive and not capable of proving a mechanism. Thus, the point of the calculations is simply to show that our results could arise from the two receptor mechanism. We are not providing experimental proof that this mechanism obtains.

We can now summarize. It appears that, if one is to look closely at the receptor kinetics of competitive blocking agents, one must consider a model in which two receptor subtypes interact both with each other and with drugs. The anesthesiologist in practice can then conclude that, although the description of the system gets more complicated when you look closely, the model still remains a straightforward extension of the classical lock-and-key analogy. The only problem is that you have to use two keys to unlock the door and anyone wanting to jam the lock can use two keys that may be different. Furthermore, the fact that the discrepancy, *i.e.*, the deviation from simple additivity, requires rather careful control of experimental conditions for its demonstration implies one can still use the classical model as a conceptual basis for clinical practice. The more complicated variant will come up more from a heuristic viewpoint or as a frame of reference when reading the literature in much the same way that it is helpful for a physician to have a general knowledge of biochemistry, although specific details may be fuzzier.

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APPENDIX

Details of the Quantitative Model

We assume a model along the lines of that of Sine and Taylor.[12] The agonist A reacts with sites R and $\underline{R}$ on each of the two alpha subunits

$$A + R \rightleftarrows AR \qquad K_A = A \cdot R/AR \tag{A1}$$

$$A + \underline{R} \rightleftarrows A\underline{R} \qquad \underline{K}_A = A \cdot \underline{R}/A\underline{R} \tag{A2}$$

(for simplicity we drop square brackets in representation of concentrations) and both sites must be occupied by A for the associated channel to open, *i.e.,* for activation. In other words, activation depends on the concentration of the form AR$\underline{R}$A. The probability that this form will occur is the product of probabilities that AR *and* A$\underline{R}$ will occur together. Thus we can write

$$\Pr(AR\underline{R}A) = \Pr(AR) \cdot \Pr(A\underline{R})$$

$$= \frac{A}{A + K_A} \cdot \frac{A}{A + \underline{K}_A} \tag{A3}$$

(again for simplicity, and without loss of generality, we set total concentration of receptor equal to unity to simplify the form of the equations).

In anticipation of a proliferation of parameters we shall introduce a somewhat arbitrary simplication; we shall set $\underline{K}_A$ equal to K_A so (A3) becomes

$$AR\underline{R}A = \frac{A}{A + K_A} \cdot \frac{A}{A + K_A} \tag{A4}$$

(While equal affinities of R and $\underline{R}$ for A will not necessarily obtain, one might still expect an agonist to be less sensitive to differences in the two receptor forms than would be an antagonist, since the latter represents an entity specialized for binding. The approximation leading to (A4) also has the *post hoc* justification that it did not obscure the final demonstration that the dimer class of model can yield predictions in excellent agreement with experiment.)

Now suppose one were to look at the effect of a competitive antagonist B with dissociation constants K_B and $\underline{K}_B$ for the reactions with R and $\underline{R}$, respectively. (Because the nature of antagonism implies a close fit to the receptor we expect, in contrast to behavior of the agonist, that K_B and $\underline{K}_B$ will in general differ. Taylor's experimental measurements confirm this.[12]) In the presence of B, K_A will be replaced[16] by $K_A(1 + B/K_B)$ for R and by $\underline{K}_A(1 + B/\underline{K}_B)$ for $\underline{R}$ so that we get

$$AR\underline{R}A = \frac{A'}{A' + K_A(1 + B/K_B)} \cdot \frac{A'}{A' + K_A(1 + B/\underline{K}_B)} \tag{A5}$$

where the prime signals the presence of the antagonist.

With two antagonists, B and B′ this will generalize to

$$AR\underline{R}A = \frac{A'}{A' + K_A(1 + B/K_B + B'/K_{B'})} \cdot \frac{A'}{A' + K_A(1 + B/\underline{K}_B + B'/\underline{K}_{B'})} \tag{A6}$$

We now must derive expressions corresponding to our experimental values. Our interaction parameter is effectively a ratio of an "observed" dose ratio produced by a mixture of two doses to one "predicted" from measurements on the drugs individually.

We shall consider the numerator of this ratio first. That dose ratio is the value of A′/A when the righthand side of (A6) is equated to that of (A4), *i.e.,* when depolarizations (or twitch responses) are matched, the values of AR$\underline{R}$A will be matched. The algebra is cumbersome but, if one writes P for $1 + B/K_B + B'/K_{B'}$, Q for its $\underline{R}$ mate and C_A for A/K_A and then solves for the dose ratio A′/A the result is

$$A'/A = \frac{2PQ/C_A}{-(P + Q) + [(P + Q)^2 + 4PQ(C_A^{-2} + 2/C_A)]^{1/2}} \tag{A7}$$

This expression is not of very meaningful appearance *per se,* but it will serve well as fuel for the computer.

Now we seek the denominator of our ratio of "observed" to "predicted" dose ratios. Here the argument is somewhat complex. What we do experimentally is measure a dose ratio, assume a simple, classical competitive model, derive a value for the corresponding K_B, repeat with $K_{B'}$, and then from B, B′, K_B, and $K_{B'}$ predict a dose ratio for a mixture. In the present context, however, we must follow through the implications of assuming the simple model if, in fact, that of (A5) were to obtain. In that latter case, when we measure our dose ratio we would be matching the righthand sides of (A5) and (A4). The solution will be of the same form as (A7), but the expressions for P and Q will lack either both second or both third terms. Given this dose ratio, one would then calculate an "apparent" K_B (or $K_{B'}$) as[16]

$$\text{"}K_B\text{"} = B/(A'/A - 1) \tag{A8}$$

In turn, one would predict[16] the dose ratio of a mixture would be

$$\text{"}A'/A\text{"} = 1 + B/\text{"}K_B\text{"} + B'/\text{"}K_{B'}\text{"} \tag{A9}$$

(A8) and (A9) can be compressed into one equivalent equation for the predicted dose ratio:

$$\text{"}A'/A\text{"}|_{\text{Mixture}} = A'/A|_{\text{B assay}} + A'/A|_{\text{B' assay}} - 1 \tag{A10}$$

In any case, the final number for comparison with our observed interaction parameter will be the ratio of the expression (A7) with that of (A9) or (A10).

There is one more practical hurdle. When we consider our more complicated model, we end up with a plethora of variables and parameters. Specifically, to solve (A7) and (A9) or (A10) we must give the computer values for A/K_A, B/K_B, $B'/K_{B'}$, $B/\underline{K}_B$ and $B'/\underline{K}_{B'}$. Five values imply a five-dimensional plot for the output. We have simplified this situation by first a reparameterization in terms of the ratios of dissociation constants

$$F = \underline{K}_B/K_B \quad \text{and} \quad F' = \underline{K}_{B'}/K_{B'} \qquad (A11)$$

Secondly, we note that typically one would use doses of B and B', which produce roughly equivalent dose ratios. Therefore, we have set up the calculation so that a value of A/K_A and dose ratio are chosen, and then values of the interaction parameter are calculated for an array of F and F' values.

Choice of a value for A/K_A is not clear. We don't have direct measures of K_A. However, precedents in other systems suggest a spare receptor capacity of the order of 100 (for example, irreversible competitive antagonists can give a dose ratio of about 100 before depressing the maximum of the dose–response curve) so we have used a value of 0.01 for A/K_A. (The results do not change much if A/K_A is made smaller. If A/K_A is 1 or greater the extent of detectable nonadditivity is blunted.)

The choice of dose ratio is easy. Measurement of the margin of safety of neuromuscular transmission[15] indicates a value of about 10 will be reasonable when the indirect twitch response is the frame of reference. On the other hand, in the present study of depolarization responses, the dose ratio was roughly 2, 3, or 4 so we can use a middle-of-the-road value of 3 for the calculations.