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The Relationship between Desensitization and the Metaphilic Effect at Cholinergic Receptors

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SUMMARY

Experiments were carried out to test the possibility that the metaphilic effect seen with certain antagonists acting at cholinergic receptors in chick and frog muscle was closely related to the phenomenon of receptor desensitization.

The action of the metaphilic antagonist dinaphthyldecamethonium mustard in blocking the contraction of chick biventer cervicis muscle or the end-plate depolarization in frog muscle was studied. In both tissues it was found that agonists that were particularly effective in desensitizing the receptors also produced a marked metaphilic effect.

A linear relationship was found between the metaphilic effect and desensitization, which was independent of the drug used to produce the desensitization. This finding was consistent with the hypothesis that metaphilic antagonists have a preferential affinity for desensitized receptors. For the antagonist studied, the rate of alkylation appeared to be about 5 times greater for desensitized than for normal receptors.

INTRODUCTION

Certain specific antagonists of drugs that depolarize the motor end-plate have recently been found to show anomalous behavior in chick and leech muscle (1). The antagonists were derivatives of decamethonium with phenyl or naphthyl groups substituted for one of the *N*-methyl hydrogen atoms at each end of the molecule, and the anomaly consisted in the finding that their potency was markedly increased if they were applied concurrently with the depolarizing drug (e.g., carbachol or suxamethonium) whose action they blocked. The evidence led us to suggest that the effect resulted from a change in the conformation of the cholinergic receptor brought about by the depolarizing drug, which increased the affinity of the blocking drug for the receptor. This proposed modification of the drug-binding properties of the receptor that was brought about by the action of depolarizing drugs was termed the metaphilic effect, and it was suggested that the same conformational change might underlie both the metaphilic effect and the phenomenon of desensitization.

In the preceding paper (2) we discussed the mechanism of desensitization to agonists in chick and frog muscle. The model that seems to account most satisfactorily for the phenomenon of desensitization is that first proposed by Katz and Thesleff (3), shown diagramatically in Fig. 1 of the preceding paper (2). This involves the formation of an agonist-receptor complex, AR, which slowly changes to a different conformational state, AR'. This complex may then dissociate, leaving the unoccupied receptor, R', in an altered conformation. R' spontaneously but slowly

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reverts to the original conformation, R. The permeability change produced by the agonist is assumed to be a function of the fraction of the receptors in the form AR, while R' represents receptors in the desensitized state.

The hypothesis that we have set out to test in this paper is that the metaphilic effect occurs because the antagonist has a higher affinity for R' than for R. In the accompanying paper it was shown that certain partial agonists were particularly effective in causing desensitization in chick and frog muscle. According to the hypothesis these drugs should produce the metaphilic effect more readily than do ordinary agonists, and this has been confirmed by results presented in this paper. Moreover, we have found a fixed relationship between desensitization and the metaphilic effect for a number of different agonists which quantitatively supports the hypothesis that the same mechanism underlies both phenomena.

METHODS

The experimental methods for recording tension from strips of chick biventer cervicis muscle, and end-plate depolarization from the extensor longus digiti IV muscle of frogs, are described in the preceding paper (2).

The salt solutions and drugs used were also the same as in the preceding paper, except that the alkylating agent decamethylene-1-(2 - chloroethyl - 1 - naphthylmethyl)amine-10 - dimethyl - (1 - naphthylmethyl)ammonium chloride hydrochloride ($DNC_{10}M$)[2, 3] was used for the measurement of the metaphilic effect. This compound is a close chemical relative of the metaphilic alkylating agent $DPC_{10}M$ tested previously (1), and had extremely similar pharmacological properties. The metaphilic effect, however, appeared to be rather more pronounced than with $DPC_{10}M$ (i.e., the effect on the blocking action of simultaneous application of a conditioning agonist was greater), and so it was preferred for this study. It was similar to $DPC_{10}M$ in its specificity and duration of action. $DNC_{10}M$ was prepared as the 2-chloroethylamine. A 10 mM solution was made up in 0.1 M HNO_3 containing 10% (v/v) ethanol. This solution (4 ml) was added to 6 ml of sodium phosphate buffer, pH 7.4, 67 mM. The opalescent mixture was kept at 37° for 30 min (during which time it partly cleared) and then kept at 0°. This procedure gave 70% conversion of the starting material to ethyleniminium ion, which was stable for several hours at 0°. The concentrations given refer to the concentration of the ethyleniminium ion.

[2] The abbreviations used are: $DNC_{10}M$, decamethylene-1-(2-chloroethyl-1-naphthylmethyl)-amine-10-dimethyl-(1-naphthylmethyl)-ammonium chloride hydrochloride; $DPC_{10}M$, decamethylene-1-(*N*-benzyl-2-chloroethylamino)-10-dimethylbenzylammonium chloride hydrochloride; C_nTMA, trimethylammonium compounds, where n = 7, 10, or 12; $C_n$bis-TMA, bistrimethylammonium compounds, where n = 13, 16, 17; phenyl-TMA, phenyltrimethylammonium iodide; C_{10}bis-DEMA, $R_1R_2R_3N^+(CH_2)_{10} \cdot N^+R_1R_2R_3 \cdot 2Br^-$, R_1, R_2 = ethyl, R_3 = methyl.

[3] The $DNC_{10}M$ used in this study was prepared by Mr. J. Ormerod and Dr. E. W. Gill, Department of Pharmacology, Oxford University.

RESULTS

Comparison of metaphilic effects produced by carbachol and $C_{10}TMA$ in chick muscle. A number of experiments were carried out in which the blocking effect of $DNC_{10}M$ was measured, with carbachol as the test agonist, after exposure of the tissue to a conditioning dose of either carbachol or $C_{10}TMA$. Figure 1 shows the results of such an experiment. The strips, taken from the same muscle, were mounted in separate organ baths. Both preparations were exposed to 7.4×10^{-7} M $DNC_{10}M$ for 10 min. In preparation *a* this was preceded by a conditioning dose of carbachol (6.6×10^{-6} M for 5 min); in preparation *b* the conditioning drug was $C_{10}TMA$ (1.1×10^{-5} M for 5 min). It can be seen that the blocking action of $DNC_{10}M$ was much greater (dose ratio, about 4) after conditioning the tissue with$C_{10}TMA$ than it was after conditioning with carbachol (dose ratio, 1.2). The concentrations of the conditioning drugs were selected to give equal, submaximal contractions of the muscle.

This difference between $C_{10}TMA$ and carbachol as metaphilic agents was investigated more thoroughly in a series of experiments whose results are summarized in Fig 2. In these experiments the conditioning agonist

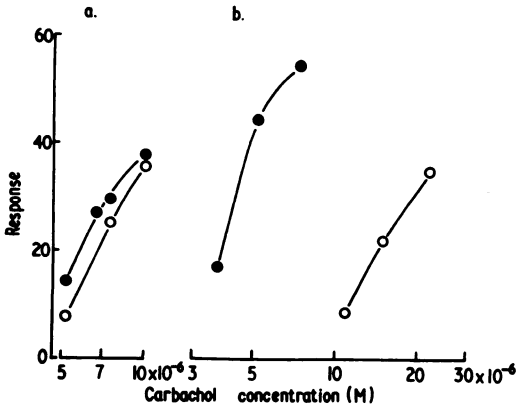

FIG. 1. *Comparison of metaphilic action of carbachol and C_{10}TMA in chick muscle*

Preparation *a*: ●, control dose-response curve to carbachol; ○, after treatment with carbachol, 6.6×10^{-6} M, for 5 min, followed by $DNC_{10}M$, 7.4×10^{-7} M, for 10 min. Preparation *b*: ●, control; ○, after treatment with C_{10}TMA, 1.1×10^{-5} M, for 5 min, followed by $DNC_{10}M$, 7.4×10^{-7} M, for 10 min. The blocking action of $DNC_{10}M$ was much greater in preparation *b*, where the conditioning drug was C_{10}TMA, than in preparation *a*, where the conditioning drug was carbachol.

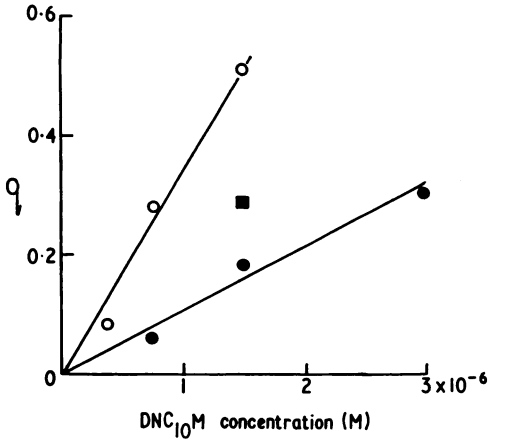

FIG. 2. *Blocking action of $DNC_{10}M$ in chick muscle.*

The fraction of receptors blocked by a 2-min exposure to $DNC_{10}M$ is plotted against the concentration of $DNC_{10}M$. Each point is the mean of two to four measurements. ●, control curve (no conditioning drug); ○, $DNC_{10}M$ applied 1 min after washing out conditioning dose of C_{10}TMA (7.4×10^{-6} M for 10 min); ■, $DNC_{10}M$ applied 1 min after washing out conditioning dose of carbachol (6×10^{-6} M for 10 min).

was left in contact with the muscle for 10 min. One minute after washing out the conditioning agonist, $DNC_{10}M$ was added and left in for 2 min. The muscle was tested with carbachol 20–30 min later to determine the amount of blockade produced by the $DNC_{10}M$. Figure 2 shows the occupancy produced by various concentrations of $DNC_{10}M$ without any conditioning agonist. In two similar experiments, the metaphilic action of equipotent concentrations of carbachol (6×10^{-7} M) and C_{10}bis-DEMA (2.2×10^{-7} M) were compared, and no difference was found. C_{10}bis-DEMA is a partial agonist, but, unlike C_{10}TMA, it does not cause a rapidly fading contraction (2). Figure 2 shows that conditioning with C_{10}TMA increased the potency of $DNC_{10}M$ by a factor of about 2.7.

Metaphilic effect in frog muscle. The metaphilic effect was readily obtained at the frog motor end plate. Thus Fig. 3 compares the blocking action of $DNC_{10}M$ when applied alone to the muscle, and when applied concurrently with a conditioning dose of carbachol. The dose ratio produced by 2×10^{-7} M $DNC_{10}M$ applied alone for 10 min was about 1.25 (i.e., 20% of the receptors were alkylated), while the same concentration resulted in a dose ratio of 2.2 (55% of the receptors alkylated) when 10^{-5} M carbachol was present for 5 min in addition to the $DNC_{10}M$. Control experiments showed (a) that the conditioning dose of carbachol did not by itself produce any long-lasting change in sensitivity, the desensitization wearing off completely within 15 min, and (b) that depolarization of the muscle by 10 mM K^+ during the application of $DNC_{10}M$ did not enhance the blocking action. The results of Hodgkin and Horowicz (4) show that bathing fluid of this composition will cause a depolarization of about 30 mV, which is about equal to the end-plate depolarization caused by 10^{-5} M carbachol (5). In our experiments the conditioning dose of carbachol was added in the presence of $DNC_{10}M$ and must have caused much less depolarization than did the addition of K^+. Since the metaphilic effect was seen only with carbachol, this rules out depolarization per se as the cause of the increased blocking action of $DNC_{10}M$.

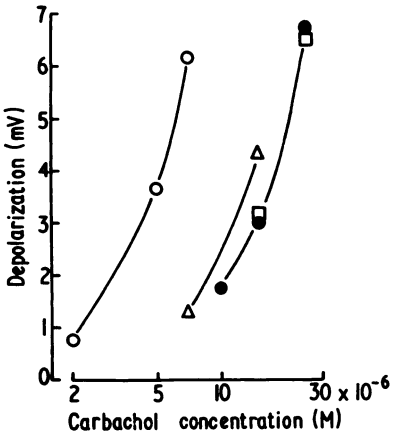

FIG. 3. *Metaphilic effect in frog muscle*

End-plate depolarization is plotted against carbachol concentration. ○, control curve; △, after $DNC_{10}M$, 2×10^{-7} M, for 10 min [carbachol (10^{-5}) M) was present as a conditioning agonist for 5 min during the exposure to $DNC_{10}M$]; ●, after a further exposure to $DNC_{10}M$ as above, but without any conditioning agonist; □, after exposure to carbachol (10^{-5} M) for 5 min, without $DNC_{10}M$. In this experiment, $DNC_{10}M$ applied on its own produced only a small antagonism (△ → ●), while the same concentration applied concurrently with carbachol had a much greater effect (○ → △). The conditioning dose of carbachol by itself had no effect (● → □).

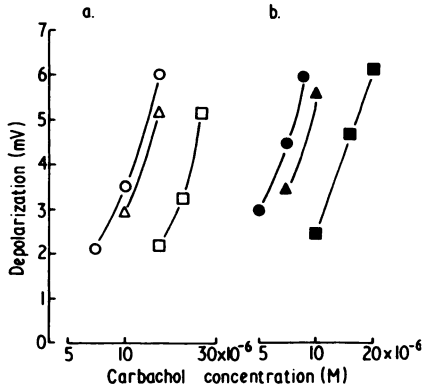

FIG. 4. *Metaphilic action of carbachol, C_7TMA, and C_{13}bis-TMA in frog muscle*

a. ○, control curve; △, after carbachol (1.5×10^{-5} M for 10 min), followed by $DNC_{10}M$ (5×10^{-7} M for 2 min) applied 1 min after washing out conditioning dose of carbachol; □, after further exposure to $DNC_{10}M$ as before, following conditioning dose of C_7TMA (2×10^{-4} M for 10 min). b. ●, control curve. ▲, after conditioning dose of carbachol (7×10^{-6} M for 10 min), followed by $DNC_{10}M$ (5×10^{-7} M for 2 min). ■, after conditioning dose of C_{13} bis-TMA (7×10^{-6} M for 10 min), followed by a further exposure to $DNC_{10}M$ as before. In each experiment, the antagonism caused by $DNC_{10}M$ after a conditioning dose of carbachol was small (○ → △, ● → ▲); the antagonism produced after conditioning with either C_7TMA or C_{13}bis-TMA was considerably greater (△ → □, ▲ → ■), though the concentrations used were equipotent in producing depolarization, with the conditioning dose of carbachol used in each experiment.

In the preceding paper (2) we reported that depolarizing agents differed in the amount of desensitization that they produced at frog motor end plates. In particular, certain agonists, such as C_7TMA, C_{13}bis-TMA, and phenyl-TMA, desensitized very much more than carbachol when the drugs were tested in concentrations that produced the same initial depolarization. The results illustrated in Fig. 4 show that the metaphilic effect produced by a conditioning dose of C_7TMA or C_{13}bis-TMA applied before $DNC_{10}M$ was added to the bath was much greater than when the conditioning agonist was carbachol. Similar results were obtained with phenyl-TMA, which was also more effective than carbachol in promoting the action of $DNC_{10}M$.

The results in frog muscle show that the metaphilic effect seen with $DNC_{10}M$ is very similar to the phenomenon in chick muscle. Drugs that cause rapidly fading depolarizations show a particularly pronounced metaphilic effect, just as in chick muscle, though different compounds are required to show the effect in the two preparations (2).

Quantitative correlation between desensitization and the metaphilic effect. The results presented so far have shown that agonists that cause marked desensitization also produce a marked metaphilic effect, and so support qualitatively the hypothesis that the metaphilic effect comes about because of a higher affinity of the antagonist for desensitized than for normal receptors. To strengthen the argument it was necessary to test the relationship quantitatively.

If the mechanism proposed for desensitization is correct, the amount of desensitization

measured at any time after a conditioning agonist is washed out of the tissue is a function of the fraction of receptors in the R' form, and this can be measured by the agonist dose ratio, as discussed in the preceding paper (2). We assume that $DNC_{10}M$, at a given concentration applied for a given length of time, will alkylate a greater fraction, q', of the receptors in the R' form, than the fraction q of receptors in the R form. If the fraction in the R' form is p_d, the over-all fraction of receptors alkylated, q_{tot}, will be

$$q_{tot} = q'p_d + q(1 - p_d)$$
$$= q + p_d(q' - q)$$

If the $DNC_{10}M$ is applied when the tissue is desensitized the over-all fraction of receptors alkylated, q_{tot}, can be measured after the desensitization has worn off. It is thus possible to estimate both q_{tot} and p_d experimentally, and to test whether the linear relationship expressed in the above equation is realized.

The experiments were done in chick muscle, as follows. A dose-response curve was obtained with carbachol. A conditioning agonist was then applied for a given length of time and then washed out, and after a given recovery period (t_r min) a test dose of carbachol, calculated to give a response in the same range as the control dose-response curve, was added. From the size of this test response, the desensitization dose ratio and, hence, p_d was calculated. The preparation was then left to recover fully. When it had done so, the conditioning agonist was applied again as before, and after t_r min $DNC_{10}M$, 1.5×10^{-6} M, was applied for 2 min. The muscle was then left for 30 min and tested with carbachol to determine the dose ratio of the blackade produced by $DNC_{10}M$ and, hence, q_{tot}. Only one such experiment could be done on each strip of muscle. A number of different conditioning agonists were tested in this way, and the recovery time was varied in a systematic way from experiment to experiment. In several experiments (see Table 1), $DNC_{10}M$ was applied without any conditioning agonist, to give an estimate of q. This varied somewhat from preparation

TABLE 1

Comparison of desensitization and metaphilic effects produced by different agonists

Conditioning agonist	Concentration	t_d[a]	t_r[b]	p_d[c]	q[d]	q_{tot}[e]
	M	*min*	*min*			
Carbachol	3.7×10^{-5}	10	3	0.38		0.39
	4.4×10^{-5}	10	2	0.46		0.50
	1.1×10^{-4}	10	3	0.51		0.54
$C_{10}TMA$	7.4×10^{-6}	10	2	0.53	0.28	0.54
	7.4×10^{-6}	10	1	0.74	0.45	0.65
	2.2×10^{-5}	10	5	0.34	0.24	0.47
	2.2×10^{-5}	10	5	0.24	0.24	0.35
	2.2×10^{-5}	10	4		0.21	0.40
	2.2×10^{-5}	10	3	0.53	0.24	0.53
	2.2×10^{-5}	10	2		0.25	0.63
	2.2×10^{-5}	10	2	0.70	0.21	0.60
	2.2×10^{-5}	10	1.5	0.85	0.26	0.67
$C_{12}TMA$	2.2×10^{-6}	11.5	3	0.48		0.50
	2.2×10^{-6}	11.5	1.5	0.62		0.54
C_{16}bis-TMA	7.4×10^{-8}	10	2	0.36		0.47
C_{17}bis-TMA	3×10^{-8}	10	2	0.80		0.64
Mean ± SE					0.25 ± 0.01	

[a] t_d = time of application of conditioning agonist.

[b] t_r = time between washing out conditioning agonist and adding $DNC_{10}M$.

[c] p_d = fraction of receptors desensitized at time t_r after washing out conditioning agonist.

[d] q = fraction of receptors blocked by $DNC_{10}M$ without any conditioning agonist.

[e] q_{tot} = fraction of receptors blocked by $DNC_{10}M$ after conditioning agonist.

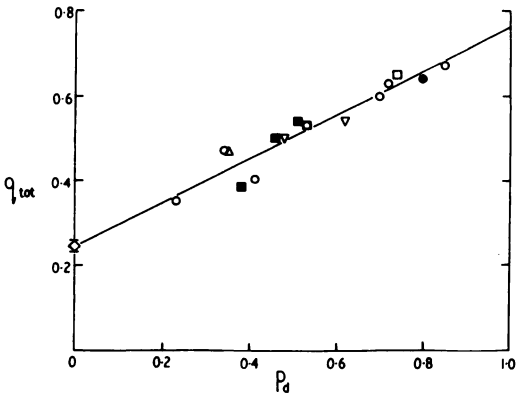

FIG. 5. *Correlation of metaphilic effect and densensitization in chick muscle*

The experimental procedure is described in the text, and the results are given in Table 1. The fraction of receptors alkylated (q_{tot}) after a standard exposure to $DNC_{10}M$ (1.5×10^{-6} M for 2 min) is plotted against the fraction of receptors desensitized. Conditioning drugs: ○, $C_{10}TMA$, 2.2×10^{-5} M; □, $C_{10}TMA$, 7.4×10^{-6} M; ■, carbachol, 4.5×10^{-5} M; △, C_{16}bis-TMA, 7×10^{-8} M; ●, C_{17}bis-TMA, 3×10^{-8} M; ▽, $C_{12}TMA$, 1.5×10^{-6} M; ◇, no conditioning agonist (mean ± standard error). The regression line was calculated by the method of least squares, and has a slope of 0.51.

to preparation, the mean value (±standard error) being $0.25 \pm 0.1(n = 11)$; this value has been plotted in Fig. 5. It can be seen from Fig. 5 that the linear relation between q_{tot} and p_d predicted theoretically was borne out by experiment. Included in these results are a series of experiments with $C_{10}TMA$ in which the recovery time after washing out the conditioning dose was varied systematically. The fact that all of these points fell on the line is evidence that the time course of recovery from desensitization is the same as the time course of decline of the metaphilic effect. Also, Fig. 5 includes results obtained with carbachol and with "desensitizing agonists" such as $C_{10}TMA$ and C_{17}bis-TMA. The relationship between desensitization and the metaphilic effect appears to be just the same in both groups, as the hypothesis demands.

A difficulty with these experiments is that the $DNC_{10}M$ was added for 2 min, during which time the desensitization would not have been constant, since the fraction of receptors in the R' form declines with a half-time of 2.3 min (2). On the other hand, the value of p_d was determined by a test dose of carbachol that was left in the bath for 2 min and produced a response that reached a plateau in about 90 sec. It seems likely that the size of this response reflects roughly the average desensitization existing during the time for which carbachol was present, and it is not unreasonable to assume that a similar average value of p_d determined the size of the metaphilic effect measured by a 2-min exposure to $DNC_{10}M$. We have assumed, therefore, that the uncertainty in the measurement of p_d and q_{tot} will not seriously distort the relationship between them.

Anomalous findings. In two experiments of the kind discussed, the conditioning drug tested was $C_{12}TMA$. This is an antagonist in chick muscle, and no stimulant action could be discerned even when the potassium concentration was raised so that the muscle developed slight tension. This procedure markedly increased the sensitivity of the muscle to carbachol, so that it would contract with about 7×10^{-7} M carbachol, whereas a concentration 3–4 times as great was needed to make it contract in normal Krebs' solution. As can be seen from Fig. 5, $C_{12}TMA$ was quite effective in producing a metaphilic effect; moreover, the size of the effect was related to the diminished sensitivity of the muscle at the time the $DNC_{10}M$ was added. Thus it appeared that the "desensitization" produced by $C_{12}TMA$ was associated with an increased affinity for $DNC_{10}M$, just as with the other drugs studied, but the desensitization occurred without any measurable stimulation of the muscle. We showed before (1) that tubocurarine did not produce a metaphilic effect, and so it is evident that occupation of the receptors is not in itself enough to enhance the blocking action of $DNC_{10}M$; the relation between the action of $C_{12}TMA$ and the desensitization produced by agonists is thus far from clear.

A second anomalous result, which is probably related to the effect of $C_{12}TMA$, was obtained when we tried to test the mechanism of the desensitizing action of $C_{10}TMA$. In the preceding paper we suggested that

C_{10}TMA caused marked desensitization because of its relatively high affinity for R'. This property might be expected to make it effective in protecting the receptors against alkylation by $DNC_{10}M$.

We therefore performed a number of experiments in which C_{10}TMA was applied as a conditioning agonist for several minutes; $DNC_{10}M$ was then applied either without washing out the C_{10}TMA or immediately after washing it out. It was argued that if C_{10}TMA were occupying a substantial fraction of R', it should protect R' against alkylation, and the $DNC_{10}M$ should have produced more blockade when it was added after the C_{10}TMA had been washed out than when it was added in the presence of C_{10}TMA. In three experiments done to test this point, the effect was not seen: the blocking action of $DNC_{10}M$ was slightly greater when it was added in the presence of C_{10}TMA than when it was added after washing out the C_{10}TMA.

DISCUSSION

The findings presented in this paper show a close correlation between desensitization and the metaphilic effect. Thus, we have found in both chick and frog muscle that agonists that are particularly effective in causing desensitization also produce a greater metaphilic effect than drugs like carbachol. This, together with the agreement between the pattern of the specificity of desensitization (2) and the production of the metaphilic effect (1), provides qualitative evidence for the hypothesis that the altered conformation of the receptors for which metaphilic antagonists have high affinity is the same as the form of the receptor responsible for the phenomenon of desensitization. The quantitative prediction of this hypothesis is that there should be a linear relationship between the fraction of the receptors alkylated by an application of $DNC_{10}M$ (q_{tot}) and the fraction of the receptors in the desensitized state when the $DNC_{10}M$ is applied (p_d), irrespective of the nature of the agonist responsible for the desensitization. This prediction was confirmed for chick muscle (Fig. 5).

From Fig. 5 it is possible to calculate roughly the relative affinity of $DNC_{10}M$ for R and for R'. In a nondesensitized muscle (assumed to contain 100% R), 25% of the receptors were aklylated in 2 min (left-hand intercept). Extrapolation to the right-hand intercept (100% R') shows that the same concentration of $DNC_{10}M$ would have alkylated 76% of R' in 2 min. If we assume that the reaction is first-order, as was found for benzilylcholine mustard (6), the half-time for alkylation of R is 4.6 min while the half-time for alkylation of R' is 0.95 min. It appears, then, that the rate of alkylation of R' by $DNC_{10}M$ is about 5 times as great as the rate of alkylation of R. This could be the result either of an increased affinity of the ethyleniminium form of $DNC_{10}M$ for R', or of a greater rate of alkylation once the reversible ethyleniminium-R' complex has been formed. Since the metaphilic effect occurs with reversible antagonists as well as alkylating agents (1, 7), the former explanation seems the more likely, though we cannot exclude the latter possibility as a contributing factor.

The anomalous results obtained with C_{10}TMA and C_{12}TMA cannot very easily be fitted into the proposed mechanism, though certain possibilities can be suggested. A common feature in the chemical structure of metaphilic agonists and antagonists is the possession of hydrophobic substituent groups, such as phenyl, naphthyl, or n-decyl. This makes it possible that hydrophobic, rather than ionic, bonding is a major factor in determining the affinity of these drugs for R'. The nature of hydrophobic interactions is such that one need not expect competition between different molecules for the binding sites. Thus binding of C_{10}TMA to a receptor might not preclude the simultaneous binding of $DNC_{10}M$. This could account for the failure of C_{10}TMA to protect the desensitized receptors against alkylation by $DNC_{10}M$. To account for the metaphilic effect of C_{12}TMA, we should have to suppose that the hydrophobic binding of C_{12}TMA actually favored the binding of $DNC_{10}M$. These are only speculative possibilities, but they suggest that the interpretation of "protection" experiments when more than

one type of binding force is involved should be treated with some caution.

The results presented in this paper provide quantitative support for the theory proposed to account for the relationship between desensitization and the metaphilic effect. The cyclic model analyzed in the preceding paper (2) is almost certainly an oversimplification, but the theory seems to account in a satisfactory way for most of the experimental results obtained.

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