Studies on the Noradrenaline α-Receptor

II. Analysis of the "Spare-Receptor" Hypothesis and Estimation of the Concentration of α-Receptors in Rabbit Aorta

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SUMMARY

The rates of recovery of response of rabbit aortic strips, blocked with *N,N*-dimethyl-2-bromo-2-phenylethylamine, to noradrenaline, adrenaline, *N*-ethyl- and *N*-isopropyl-noradrenaline have been determined. The close similarities of these recovery rates suggests that "spare" receptors are of little significance in this system.

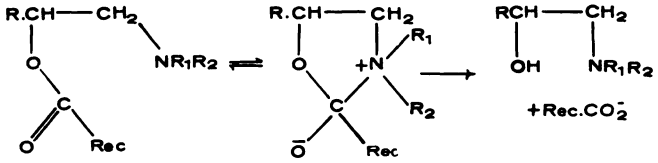
The use of ^{3}H-labeled *N,N*-dimethyl-2-bromo-2-phenylethylamine enabled an estimate of the concentration of α-receptors, 1.15×10^{12} receptors per milligram of tissue dry weight, to be made and also provided good evidence for the view that tissue response is proportional to the concentration of agonist–receptor complex.

In Part I (1) of this projected series of papers dealing with the structure of the adrenergic α-receptor we discussed the specificity of action of *N*-(2-bromoethyl)-*N*-ethyl-*N*-1-naphthylmethylamine hydrobromide (SY.28, I),[1] an agent representative of the competitive irreversible antagonists active at the α-receptor. From this discussion it was apparent that theoretical schemes, based on protection of the receptor with an agonist or short-lasting antagonist prior to treatment with labeled blocking agent, were unlikely to be successful with the irreversible blocking agents currently available (1). The reasons for this are the relative lack of specificity of the irreversible antagonists and of noradrenaline itself. Thus noradrenaline and other sympathomimetic agents are known to possess substantial affinity for sites other than the adrenergic α-receptor; of particular importance among such "nonreceptor" sites are the amine uptake and storage sites (2–6) and among other sites may be listed monoamine oxidase, catechol-0-methyltransferase and other less well defined "sites of loss" such as serum albumin (7). It will be anticipated that the 2-halogenoethylamines, whose structure-activity relationship can be rationalized in terms of the phenylethylamine pattern (8) characteristic of sympathomimetic amines, will also show affinity for some or all of these sites (9, 10), in addition to their known pharmacologic activities in nonadrenergic systems (11–14). These factors together with the very low concentration of receptor material in tissues makes the interpretation of protection data of the variety discussed in Part I very difficult. It thus appeared desirable to attempt to obtain an experimental estimate of the concentration of adrenergic α-receptor material in tissues. The present paper records our approach to this problem.

From our previous discussion (1), it was apparent that the method adopted should involve the use of physiologically viable tissue. For this reason, it was decided not

[1] Abbreviations used: SY.28, *N*-(2-bromoethyl)-*N*-ethyl-1-napthylmethylamine; II, *N,N*-dimethyl-2-bromo-2-phenylethylamine.

to adopt any procedures which involved the determination of noradrenaline or irreversible antagonists bound to tissue fractions or to determine by autoradiographic techniques the sites and amounts of agonist or antagonist bound in various tissues. The technique that was adopted is based upon our previous examination (9) of the kinetics of recovery of the response to noradrenaline of rabbit aortic strips pretreated with the irreversible competitive antagonist, *N,N*-dimethyl-2-bromo-2-phenylethylamine (II). In this work it was demonstrated that II produced an irreversible block of rather short duration and that the recovery process obeyed first-order kinetics. From our previous and unpublished observations on the duration of adrenergic blocking action of 2-halogenoethylamines, an explanation of the relative rates of recovery of response from blockade by these agents is best accommodated in terms of an intramolecularly facilitated hydrolysis of the alkylated receptor, provided that certain assumptions are made concerning the relationship between receptor occupancy and tissue response. Strictly speaking, our work on the duration of action of 2-halogenoethylamines refers only to the rate of recovery of tissue response, not to the rate of receptor regeneration. An interpretation in terms of the latter can be made if it is assumed that tissue response is proportional to receptor occupation and that maximum tissue response occurs with 100% receptor occupancy (9). These are, of course, the fundamental assumptions made by Clark in his original quantitative treatment of drug-receptor interactions (15).



With the above assumptions a determination of the amount and time course of the loss of radioactivity from tissues treated with ^{3}H-*N,N*-dimethyl-2-bromo-2-phenylethylamine would provide an estimate of the amount of α-receptor material in tissues. This would certainly be a maximum figure because of the relative nonspecificity of action of 2-halogenoethylamines. If it is assumed that the site alkylated at the α-receptor is a carboxylate anion, a certain number of nonreceptor carboxylate anions will also be alkylated and thus may contribute to an overestimation of the amount of α-receptor material. The importance of this factor may, however, be reduced by appropriate control experiments (see Results and Discussion).

However, the assumptions made originally by Clark have, in recent years, been subject to serious challenge by Ariëns, Stephenson, Furchgott and their co-workers (16–20).[2] Essentially, these workers have suggested that tissue response may not be proportional to the concentration of drug-receptor complex (receptor occupancy) and that maximum tissue response can occur when only a small percentage of the receptors are occupied (the "spare" receptor hypothesis). Estimates of the percentage of receptors that have to be occupied to produce a maximum response have ranged down to 0.0001% (21), although it is proposed that the percentage occupancy at maximum response is a function of the structure of the agonist.

[2] An alternative treatment has been offered by Paton (22, 23) which differs rather substantially from the Clark-Ariëns-Stephenson model since it does not require the formation of a discrete intermediate drug-receptor complex for receptor activation to occur. The act of association of a molecule with the receptor is presumed sufficient to initiate tissue response. The foundations of this "rate" theory of drug action have been subject to very pertinent criticism by Belleau (24), and at the present time it would seem more profitable to consider drug-receptor interactions on the basis of the formation of a discrete intermediate complex as proposed in the Clark-Ariëns-Stephenson model (16).

Clearly, acceptance of the concepts of "spare" receptors and of a lack of proportionality between tissue response and concentration of drug-receptor complex would invalidate any interpretation of our data for the rates of recovery of tissue response following exposure to 2-halogenoethylamines, in terms of receptor structure, since it might be argued that our observations of 100% recovery of tissue response refers only to reactivation of, for example, 0.1% of the total receptor population (9). It is equally apparent that attempts to determine the concentration of receptor material in tissues by the method mentioned (page 29) would be subject to the same criticism. Before attempting to carry out the latter experiment, it appeared desirable, therefore, to test the validity of the assumptions made by Clark, Ariëns, Stephenson, *et al.*

A certain amount of pertinent criticism, at the theoretical level (9, 24), has been directed at the Clark-Ariëns-Stephenson model of drug-receptor interaction. In this paper we present experimental evidence bearing on this model and which appears to be of value in formulating theories of drug-receptor interaction.

MATERIALS AND METHODS

Pharmacology. Rabbit aortic strips were set up to record isotonic contractions (25, 26). Doses of the drugs are in grams per milliliter (final bath concentration) and refer to the following salts: DL-*N*-ethylnoradrenaline·HCl, DL-isopropylnoradrenaline·HCl, D(—)-noradrenaline bitartrate, DL-adrenaline as the free base, SY.28 and *N,N*-dimethyl-2-bromo-2-phenylethylamine as the hydrobromides.

Release of radioactivity. Strips were completely blocked to noradrenaline with ^{3}H-*N,N*-dimethyl-2-bromo-2-phenylethylamine after which they were washed repeatedly for 10 minutes. The volume of the bath was then returned to 20 ml and 50-μl aliquots were taken at 5-min intervals and counted in the scintillator solution described earlier (1). A minimum of 10,000 counts was collected for each sample and corrections for quenching were made by the method of internal standardization (27). The first-order rate constant was determined by plotting the data according to Rose (28). Briefly, the alternate values of total radioactivity in the bath are plotted against one another, i.e. the first 5 min determination against the third, the second against the fourth, etc. The logarithm of the slope of the straight line multiplied by $2.303/\Delta t$, where $\Delta t = 10$ min, gives k in accordance with the equation

$$k = \frac{2.303}{\Delta t} \log \text{slope}: t_{1/2} = \frac{0.693}{k}$$

From the $t_{1/2}$ it is possible to determine the fraction of radioactive material appearing in the bath over the total period of measurement and hence, to calculate the total amount of radioactivity which would appear in the bath fluid if the process were followed to completion. Usually measurements were made over a period of 1 hr (approximately 3 half-lives) after which the strips were counted as described previously (1), and the fraction representing material which was covalently bound and which would not appear in the radioactive washout process was determined by subtracting the calculated value of the material remaining which would undergo hydrolysis on the basis of the first-order rate process. Independent rate constants were determined in parallel strips using noradrenaline, adrenaline, *N*-ethylnoradrenaline, and *N*-isopropylnoradrenaline and treating the data as described previously (9, 26).

Chemical synthesis of labeled material N,N-dimethyl-2-bromo-2-phenyl-(2-^{3}H) ethylamine hydrobromide, PhC^{3}HBrCH$_2$NMe$_2$, HBr. To ω-*N,N*-dimethylaminoacetophenone (29) (1.80 g, 0.011 mole) dissolved in methanol (30 ml) and stirred magnetically at 15–20° was added tritiated sodium borohydride (114 mg, 0.003 mole, specific activity 200 mC/mmole from New England Nuclear Corporation) dissolved in 2 ml of 5% sodium hydroxide. After 2 hr stirring a further 30 mg of unlabeled sodium borohydride was added and most of the solvent was removed by dis-

tillation. The oil was taken up into chloroform (2 × 5 ml) and dried over anhydrous magnesium sulfate. The dried chloroform extract was stirred at 0° and a solution of phosphorus tribromide (3.0 g) in chloroform (8 ml) was added dropwise. When the addition was complete the mixture was refluxed for 4 hr. The solvent was removed, and the residue was treated with boiling methanol (2 × 25 ml) and filtered. On cooling, the filtrate yielded *N,N*-dimethyl-2-bromo-2-phenyl-(2-^{3}H)ethylamine hydrobromide (2.5 g, 81%), m.p. and mixed m.p. 175–176°. The material was recrystallized to constant specific activity and demonstrated to be pure by reverse isotope dilution. The specific activity was 41.6 mC/mmole.

RESULTS

Responses of Aortic Strips to Catecholamines

Dose-response curves for noradrenaline, adrenaline, *N*-ethylnoradrenaline, and *N*-isopropylnoradrenaline were determined using rabbit aortic strips set up according to the method described by Furchgott and Bhadrakom (25). The results have been expressed in terms of the intrinsic activity factor—maximum contraction height of agonist/maximum contraction height of noradrenaline (26). It will be observed that noradrenaline and adrenaline have equal intrinsic activities, i.e., they produce the same maximum contraction, but *N*-ethyl- and *N*-isopropylnoradrenaline produce maximum contractions which are 90% and 60%, respectively, of that produced by noradrenaline. These results are in good agreement with previously published data (30) (Table 1).

TABLE 1

The activities of catecholamines in α-receptor systems

Cumulative dose response curves were determined on rabbit aortic strips with noradrenaline followed by determination of the response of the same strips to the other catecholamines. Decreases in sensitivity were determined on control strips taken from the same animal.

	Intrinsic activity[a]	
Amine	Rabbit aorta	Rat vas deferens (data from 30)
DL-Adrenaline	1.0	1.0
D(−)-Noradrenaline	1.0	1.0
DL-*N*-Ethylnoradrenaline	0.9	0.9
DL-*N*-Isopropylnoradrenaline	0.6	0.6

[a] Intrinsic activity = $\frac{\text{maximum response agonist}}{\text{maximum response to noradrenaline}}$

Rates of Recovery of Tissue Response Following Blockade by N,N-Dimethyl-2-bromo-2-phenylethylamine

Rabbit aortic strips were completely blocked to noradrenaline by pretreatment with *N,N*-dimethyl-2-bromo-2-phenylethylamine (5×10^{-6} g/ml for 5 min) and the rates of recovery of tissue response to a series of agonists, noradrenaline, adrenaline, *N*-ethylnoradrenaline, and *N*-isopropylnoradrenaline, were determined. Full details of the method used in plotting the recovery data have been given previously (9, 26). The only modification made in these experiments was to employ an amount of the stimulating agonist that was marginally above the ED_{95} determined from internal controls. This procedure made possible a

TABLE 2

The half-life of α-receptor blockade by N,N-dimethyl-2-bromo-2-phenylethylamine as measured by the response to agonists, partial agonists, and release of radioactivity

The $t_{1/2}$'s were determined from the first-order plots as described in Materials and Methods.

Compound	$t_{1/2}$ (min) ± S.D.
DL-Adrenaline (7)[a]	19.7 ± 5.8
D(−)-Noradrenaline (7)	23.0 ± 2.6
DL-*N*-Ethylnoradrenaline(7)	26.6 ± 4.4
DL-*N*-Isopropylnoradrenaline (6)	27.8 ± 3.2[b]
^{3}H-*N,N*-Dimethyl-2-bromo-2-phenethylamine (8)	20.7 ± 4.1

[a] Numbers in parentheses refer to number of experiments.

[b] Significantly different from noradrenaline using the student *t* test, $p = <0.05$.

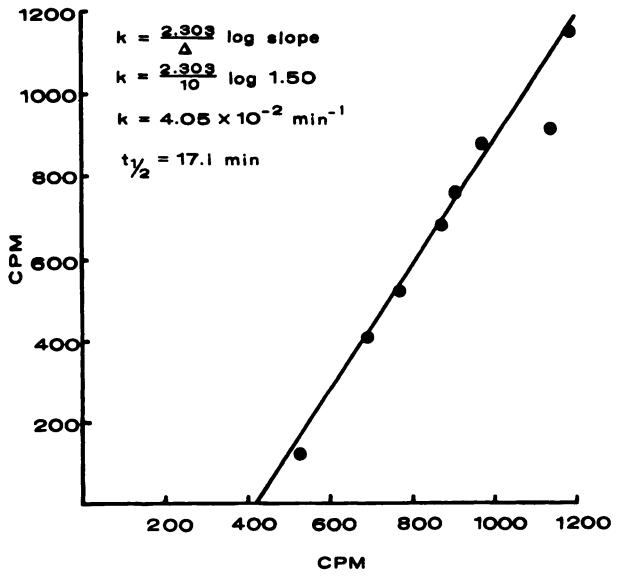


FIG. 1. *Rate of release of tissue radioactivity plotted according to Rose (28)*

TABLE 3

Estimation of the number of α-receptor sites in rabbit aortic strips

Amount of ^{3}H-*N*,*N*-dimethyl-2-bromo-2-phenylethylamine, sufficient to give 100% blockade as measured by the pharmacologic response to a supramaximal dose of noradrenaline, were incubated with rabbit aortic strips for 5 min in 20-ml muscle baths. After successive washing for 10 minutes, the volume was set at 20 ml and 500-μl samples were taken. The radioactivity was determined and data were treated as described in Materials and Methods.

Entry	Conc. of II (μg/ml)	Total Dpm/mg appearing in wash fluid		Dpm/mg remaining on strip	$t_{1/2}$ (min)	Number of molecules appearing in wash fluid/mg dry weight tissue ≡ No. receptors/mg dry weight tissue	
		Control	SY.28[a]				
1	1.8	503	—	1054	24	3.3×10^{12}	3.6×10^{12}
2	1.8	719	—	1016	20	4.7×10^{12}	
3	1.8	483	—	1252	29	3.2×10^{12}	
4	1.8	509	—	1024	17	3.35×10^{12}	
5	1.8	—	330	774	29	2.2×10^{12}	$\frac{2.45 \times 10^{12}}{1.15 \times 10^{12}}$
6	1.8	—	416	610	24	2.7×10^{12}	
7							
8	3.7	951	—	2000	21	6.3×10^{12}	7.0
9	3.7	1172	—	2250	20	7.7×10^{12}	
10	3.7	—	669	1320	27	4.4×10^{12}	$\frac{4.25}{2.75 \times 10^{12}}$
11	3.7	—	628	1054	24	4.1×10^{12}	
12							
13	5.6	984	—	2611	17	6.4×10^{12}	
14	5.6	930	—	1832	18	6.1×10^{12}	

[a] Prior to treatment with ^{3}H-*N*,*N*-dimethyl-2-bromo-2-phenylethylamine these tissues were preblocked with SY.28 (5×10^{-6} g/ml for 10 min).

more accurate determination of initial rates and has resulted in a minor change from our previously published first-order rate constant for recovery to noradrenaline. The results of these experiments are presented in Table 2. First-order kinetics of recovery was observed in all cases.

Rates and Amount of ^{3}H-Washout Following Blockade by ^{3}H-N,N-Dimethyl-2-bromo-2-phenylethylamine

In experiments similar to those described above, ^{3}H-*N*,*N*-dimethyl-2-bromo-2-phenylethylamine was employed. Rabbit aortic strips were blocked 100% with the minimum concentration of this agent and were then washed thoroughly for 10 min to remove noncovalently bound material. In control experiments we found this time sufficient to wash out completely an equivalent amount of the corresponding alcohol. The strips were then stimulated at 20-min intervals with noradrenaline (ED_{95}, see Table 2) or, in other strips, the radioactivity lost from the tissue in each 5-min period was determined. Thus records of tissue recovery and radioactive loss were obtained from strips from the same animal. A Guggenheim-type plot (28) of the radioactivity data gave first-order plots (Fig. 1 is representative) with the $t_{1/2}$ for the process (Table 2) being 20.7 ± 4.1 min.

Since our previous work with 2-halogenoethylamines indicates that they are relatively nonspecific blocking agents, it was of interest to determine the total amount of radioactivity coming from tissues that had been treated with concentrations of ^{3}H-*N*,*N*-dimethyl-2-bromo-2-phenylethylamine somewhat higher than required for 100% blockade. These results are presented in Table 3. In other experiments designed to explore this point further, aortic strips were pretreated with SY.28 (a long-lasting irreversible α-blocker, see Part I) to produce complete blockade of the response to noradrenaline prior to treatment with ^{3}H-*N*,*N*-dimethyl-2-bromo-2-phenylethylamine. The results of these experiments are also presented in Table 3.

DISCUSSION

In the series of compounds noradrenaline, adrenaline, *N*-ethylnoradrenaline, *N*-isopropylnoradrenaline, noradrenaline and adrenaline are full agonists and *N*-ethyl- and *N*-isopropylnoradrenaline are partial agonists in the Ariëns-Stephenson terminology (31). That is, *N*-ethyl- and *N*-isopropylnoradrenaline do not produce the maximum responses (relative to the maximum response of noradrenaline) even at maximum concentrations (Table 1). According to the Ariëns-Stephenson theory, the phenomenon of spare receptors is not observed with partial agonists because such agents have to occupy all the receptors in order to produce their maximum responses.[3] Thus, determination of the rates of recovery from irreversible blockade by *N*,*N*-dimethyl-2-bromo-2-phenylethylamine using both full and partial agonists as the stimulating agents should give either essentially identical recovery rates if spare receptors do not exist and the agonists have to occupy the same number of receptors to produce their respective maximum response, or widely differing rates of recovery if spare receptors have the importance that the Ariëns-Stephenson model would suggest. From the results in Table 2, it is apparent that the differences in the rates of recovery of tissue response with the various agonists are far smaller than would be anticipated if a significant concentration of spare receptors existed. Thus, if noradrenaline, which shows a $t_{1/2}$ of 23 ± 2.6 min, needed to occupy only 0.1% of the α-receptors to produce a maximum response then the $t_{1/2}$ for *N*-isopropylnoradrenaline which, according to the Ariëns-Stephenson treatment, has to occupy 100% of the receptors to produce

[3] The phenomenon of "spare" receptors is also encountered in the Paton theory of drug action in the form of "spare receptor capacity," where it is proposed that if the rate of dissociation of the agonist from the receptor is sufficiently rapid, then maximum response is determined by the properties of the effector system rather than by the kinetics of the drug-receptor interaction.

its maximum response, would be greater than 20 hr as compared to the observed time of 27.8 ± 3.2 min. The close similarity in the times for 50% recovery listed in Table 2 indicates that all four agonists have to occupy approximately the same number of receptors to produce their respective maximum responses,[4] and that, at least for the rabbit aortic strip system, spare receptors are of little consequence.[5]

Thus, to produce the same percentage response of their respective maximum responses, noradrenaline, adrenaline, *N*-ethylnoradrenaline and *N*-isopropylnoradrenaline have to occupy approximately the same number of receptors. It is apparent however, that, since *N*-isopropylnoradrenaline produces a maximum response which is only 60% of that produced by noradrenaline (Table 1), then 40% of the *N*-isopropylnoradrenaline-receptor interactions do not lead to tissue response. (An argument strongly supporting the concept that tissue-response is proportional to the concentration of agonist-receptor complex is presented in the next section.) This conclusion accords well with Belleau's conformational perturbation theory of drug-receptor interactions (24) in which he concludes that both specific and nonspecific conformational changes of the receptor can occur following interaction with a drug molecule, but that only the former changes can lead to tissue response.

Further evidence of direct relevance to these conclusions was obtained from the experiments in which the aortic strips were completely blocked with the minimum concentration of ^{3}H-*N*,*N*-dimethyl-2-bromo-2-phenylethylamine. The $t_{1/2}$ for the loss of tissue radioactivity, which we assume to represent material from the hydrolysis of alkylated receptor, was found to be 20.7 ± 4.1 min (Table 2) which is in good agreement with the $t_{1/2}$ for the pharmacologic recovery process which was measured simultaneously. The near identity of the kinetic constants of these processes certainly suggests that the processes of recovery of response and of regeneration of the α-receptor are directly related. If this conclusion is accepted then it follows that, to a close approximation, the magnitude of the agonist-induced response in rabbit aortic strips is proportional to the concentration of agonist-receptor complex. However, the results in Table 3 indicate that as the concentration of ^{3}H-*N*,*N*-dimethyl-2-bromo-2-phenylethylamine is raised above the minimum blocking dose, so the amount of radioactivity coming from the tissues tends to increase, suggesting the increased importance of release from nonreceptor material. In other experiments in which aortic strips were pretreated with SY.28 to produce 100% blockade of the α-receptors, prior to treatment with ^{3}H-*N*,*N*-dimethyl-2-bromo-2-phenylethylamine, a 25–40% reduction in radioactive washout was observed; however, the $t_{1/2}$ for the remaining, nonreceptor, process was not significantly different from that observed in control strips (Table 3).[6] This result is not surprising if it is assumed that the site alkylated at the adrenergic

[4] We are currently examining the application of this approach to other tissue systems with the dual aim of extending the validity of this conclusion and of improving the accuracy of our results.

[5] It will be observed, however, that there is a definite trend in the times for 50% recovery listed in Table 2, and this trend tends to parallel the increase in β-mimetic activity of the catecholamines. Thus the difference in rates of recovery between noradrenaline and *N*-isopropylnoradrenaline is statistically significant. However, even if it is assumed that all this difference represents spare receptors, then it can be calculated that at maximum response with noradrenaline there would be only 14% spare receptors. It seems equally plausible, however, that the slower recovery rates found with *N*-isopropylnoradrenaline, and to a lesser extent with *N*-ethylnoradrenaline, are due to the increased activities of these compounds, relative to noradrenaline and adrenaline, at the β-receptors (32).

[6] It is of interest to note from the results in Table 3, that strips pretreated with SY.28 were protected against nonspecific and specific labeling (amount released by hydrolysis and amount remaining bound to tissue) by II to approximately the same extent. This confirms our previous findings (1) that SY.28 is chemically relatively nonspecific.

α-receptor is a carboxylate anion (8, 9, 26), since its occurrence in nonreceptor material must be relatively common, i.e., glutamyl and aspartic acids, C-terminal amino acids, fatty acids, and polysaccharides.

The difference in washout of ^{3}H-material between the SY.28 pretreated tissues (Entries 5, 6, 10, and 11, Table 3) and the control tissues (Entries 1, 2, 3, 4, 8, and 9, Table 3) represents material derived, in part, from hydrolysis of the ^{3}H-labeled receptor and hence, assuming one alkylation per α-receptor, gives a maximum estimate of the number of α-receptors per unit of tissue (Entries 7 and 12, Table 3). The figure calculated from experiments using the minimum blocking concentration of ^{3}H-*N,N*-dimethyl-2-bromo-2-phenylethylamine, 1.15×10^{12} receptors per milligram of tissue dry weight, should be the most accurate estimate although it must be emphasized that this represents a maximum figure because the relevant ^{3}H-washout data probably contain a contribution from the hydrolysis of nonreceptor material.

Since there does not appear to have been any other substantial attempt to determine adrenergic α-receptor concentrations we are unable to present any comparison with our data for this system. However, it appears of interest to compare our results with estimates of receptor concentration in other systems.

Trams (33) has calculated from the data of Waser (34) and others (35) that tissue from the electric organ of the electric eel can bind 1.12 mμmole curare per gram of tissue, which is equivalent to 3.4×10^{12} receptor sites per milligram of tissue dry weight. Paton and Rang (23) have studied the uptake of atropine in guinea pig intestinal smooth muscle and have tentatively identified one component of this uptake, with a binding capacity of 1.8×10^{-10} mole/g, with the cholinergic receptor. This corresponds to a figure of approximately 5×10^{11} acetylcholine receptors per milligram of tissue dry weight. The agreement, to within an order of magnitude, of the figures for the tissue concentrations of adrenergic and cholinergic receptors is of interest, although probably fortuitous, since it must be recognized that these results were obtained by different techniques with different tissues.

Furthermore, the technique of receptor titration and isolation using competitive reversible antagonists offers serious drawbacks which the use of an appropriately designed irreversibly acting antagonist can avoid. It seems probable that the use of such irreversibly acting agents may lead to significant advances in this field. The approach reported in this paper has served to suggest the insignificance of "spare" receptors in rabbit aortic strips and to demonstrate a proportionality between tissue response and the concentration of agonist–receptor complex. Furthermore, our approach has made possible an estimation of the quantity of α-receptor material in an adrenergically innervated tissue. This figure is certainly a *maximum* estimate, but it does serve to give an indication as to the sensitivity of methods and the amounts of material required for an effective receptor isolation procedure.

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