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THE BINDING OF [^{3}H]-PROPYLBENZILYLCHOLINE MUSTARD BY LONGITUDINAL MUSCLE STRIPS FROM GUINEA-PIG SMALL INTESTINE

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1 The synthesis of tritium labelled propylbenzilylcholine mustard ([^{3}H]-PrBCM; *N*-2′-chloroethyl-*N*-[2″, 3″-$^3H_2$] propyl-2-aminoethyl benzilate) is described.

2 The uptake by muscle strips was measured and shown to be considerably increased by previous immersion of the muscle in distilled water.

3 A considerable part of the uptake is inhibited selectively by atropine, but not by nicotinic antagonists. A number of muscarinic agonists also inhibit uptake and their apparent affinity constants have been determined.

4 The uptake by atropine-sensitive sites is temperature-insensitive, whereas the other sites are temperature-sensitive. Recovery is highly temperature-sensitive and there is good agreement between recovery of sensitivity to agonists and loss of radioactivity from the muscle.

Introduction

Benzilylcholine mustard, which was introduced by Gill & Rang (1966), is an alkylating agent with considerable specificity for muscarinic receptors and is a candidate for use as an affinity label (Rang, 1967; Fewtrell & Rang, 1971). In a previous communication (Young, Hiley & Burgen, 1972), we described the synthesis of a series of homologues of benzilylcholine mustard (BCM) and showed that the *N*-propyl homologue (PrBCM) was about equiactive. PrBCM has the advantage as an affinity label that it is easier to introduce tritium in high specific activity and known position. In this paper, we describe the preparation of [^{3}H]-PrBCM and some of its properties as an affinity label for muscarinic receptors in smooth muscle of the guinea-pig ileum.

Methods

Synthesis of N-2′-chloroethyl-N-[2″,3″-$^3H_2$] propyl-2-aminoethyl benzilate ([^{3}H]-PrBCM)

[^{3}H]-PrBCM was synthesized by reduction of the corresponding allyl derivative with tritium gas in the presence of a palladium catalyst (Figure 1). The use of palladium rather than platinum avoids hydrogenolysis of the allyl residue but the reaction mixture still contains a second product, as yet unidentified, in addition to [^{3}H]-PrBCM. These two products are not readily separated when present in approximately equal amounts, and we have therefore resorted to an isotopic dilution technique in order to extract the [^{3}H]-PrBCM.

N-allyl-*N*-2′-chloroethyl-2-aminoethyl benzilate (21.4 mg) (Young *et al.*, 1972) in dioxan (0.6 ml dried over CaH_2) was hydrogenated at room temperature with tritium gas (2 Ci, i.e. 0.8 cc at N.T.P.) in the presence of 5% palladium on charcoal (5 mg) in an apparatus similar to that used by Glascock (1954). After 4 h any tritium gas remaining was removed and the reaction mixture filtered. The reaction vessel and filter were washed with warm ethanol and the combined filtrate and washings treated with PrBCM (0.13 g), dissolution being achieved by gentle warming. A little dry ether was added, the small amount of dirty precipitate which formed filtered off, and the filtrate then treated (with more ether) to incipient precipitation (no permanent cloudiness). The [^{3}H]-PrBCM hydrochloride (yield 85 mg) which crystallized out over three days at room temperature had a specific activity of 1.45 Ci/mmol, which was not increased by a further recrystallization. The product was stored at -10° C as a 6.5 mM ethanolic solution.

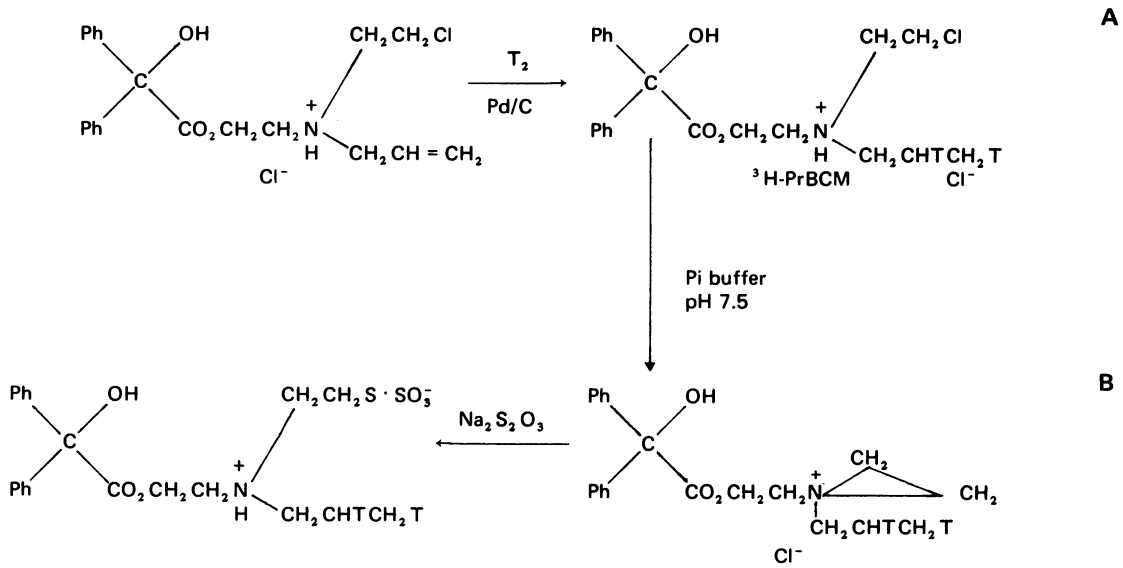

Fig. 1 Scheme for synthesis of [^{3}H]-propylbenzilylcholine mustard.

Radiochemical purity

The specific activity of [^{3}H]-PrBCM remained constant on recrystallization—evidence that the tritium is present as [^{3}H]-PrBCM rather than as a small amount of a highly radioactive impurity. As a further check on the radiochemical purity we have utilized the fact that only the chloroethyl compound cyclizes in aqueous solution to form an aziridinium ion derivative, which reacts quantitatively with thiosulphate (Golumbic, Fruton & Bergmann, 1946) yielding a product which, at pH 3.0, carries one positive and one negative charge. The parent chloroethylamine has one positive charge only. We have therefore examined the behaviour of [^{3}H]-PrBCM on electrophoresis at pH 3 (a) before cyclization and (b) after being allowed to cyclize at pH 7.4, followed by reaction with sodium thiosulphate.

More concentrated samples of non-radioactive PrBCM were treated in the same way and run in parallel. After electrophoresis the non-radioactive spots were stained with iodine vapour and the two tracks with the tritiated compound scanned for radioactivity (Figure 2). The peaks corresponded exactly with the iodine spots on the appropriate non-radioactive control. The change in the mobility is consistent with the presence of the tritium only as [^{3}H]-PrBCM.

The major peak in the cyclized and thiosulphate treated sample, the alkylthiosulphate derived from the aziridinium ion, represented 87% of the total radioactivity, which agrees closely with the yield of 85-90% from the cyclization reaction under these conditions (Young *et al.*, 1972).

Formation of the aziridinium ion derivative

The pharmacologically active species is the aziridinium ion formed in aqueous solution at a pH such that the parent compound is largely in the free base form. [^{3}H]-PrBCM was routinely cyclized at a concentration of 0.13 mM in 10 mM phosphate buffer, pH 7.6 for 1 h at room temperature and the reaction stopped by diluting an aliquot 1 : 100 in ice cold buffer. For convenience in describing additions, the solution added is termed [^{3}H]-PrBCM; but this always refers to the cyclized reaction mixture and the concentration quoted is that of the aziridinium derivative calculated on the basis of a 91% yield.

Previously, the cyclization reaction has been carried out at concentrations around 1 mM so as to yield amounts of the aziridinium ion which could be conveniently assayed titrimetrically. However, the yield of the aziridinium ion is less than quantitive, mainly because of hydrolysis but possibly also because the aziridinium ion once formed may react with the parent chloroethylamine, which, in the uncharged form, is a good nucleophile. This side-reaction, a second order process, should be slowed as the concentration of [^{3}H]-PrBCM is reduced, while the first-order cyclization should be unaffected. We have therefore determined the yield of the

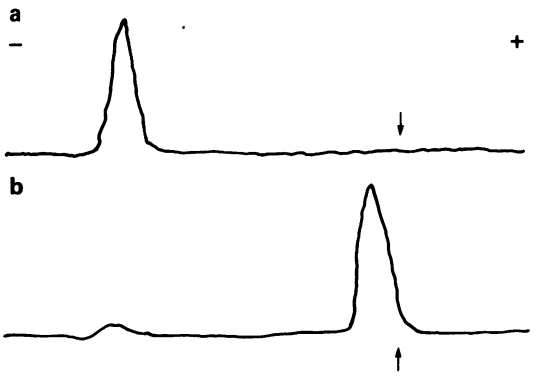

Fig. 2 Electrophoretic test of the radiochemical purity of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM). (a) 0.13 mM [^{3}H]-PrBCM in 60 mM citric-acid-Na_2HPO_4 buffer, pH 3.05. (b) 0.13 mM [^{3}H]-PrBCM in 10 mM phosphate buffer, pH 7.4. Both samples were allowed to stand for 60 min at room temperature (~22° C), treated with 0.1 ml 10 mM thiosulphate and allowed to stand a further 45 min before dilution to 5 ml with 60 mM citric-acid-Na_2HPO_4 buffer, pH 3.05. Electrophoresis was carried out on Whatman No. 1 paper in the citric-acid-phosphate buffer for 4 h 45 min at room temperature, voltage drop 7.5 V/cm. The traces are scans for radioactivity (Packard Model 7200 Radiochromatogram scanner) after drying at 100° C.

aziridinium ion, using the electrophoretic method described above, as the concentration of [^{3}H]-PrBCM was varied from 2.14 mM down to 0.07 mM. At the highest concentrations, the higher mobility (aziridinium ion) peak showed a distinct shoulder. Reducing the concentration of [^{3}H]-PrBCM removed this shoulder, but the improvement in the yield, from 84% to 91%, was small. The maximum yield was obtained with [^{3}H]-PrBCM concentration of 0.2 mM and less.

Determination of the binding of [^{3}H]-propylbenzilylcholine mustard by strips of longitudinal muscle from guinea-pig small intestine

Strips of longitudinal muscle from guinea-pig small intestine, usually weighing between 9 and 17 mg, were prepared as described by Rang (1964) from animals of either sex normally weighing 500-750 g. The strips were suspended under their own weight in Krebs-Henseleit solution by means of capillary attraction between a thread tied round one end and the walls of the incubation vessel. The volume of incubation medium per strip varied between 30 and 60 ml. Stirring was effected by bubbling with 95% O_2 and 5% CO_2.

The strips were preincubated for 1 hour. Any antagonists to be tested were added at this time and 30 min later, [^{3}H]-PrBCM was added to a final concentration of 2.4 nM. Uptake was terminated by transferring the strips to fresh Krebs solution (200 ml). Washing was continued for 75 min at 30° C with five changes of Krebs solution and the strips were then stored in Krebs solution at 0° C. Each strip was blotted dry on Whatman No. 1 filter paper and transferred to a plastic scintillation vial for weighing. Soluene (Packard) (0.7 ml) was added and the vials allowed to stand overnight. This procedure sufficed to dissolve most strips completely; any aggregates remaining were readily dispersed by gentle agitation. Ethoxyethanol (1 ml) and 0.4% butyl PBD in toluene (10 ml) were added and the tritium determined in a Packard Tricarb Model 3320 liquid scintillation counter.

Results

Binding of [^{3}H]-propylbenzilylcholine mustard by longitudinal muscle strips

The time course of the binding of [^{3}H]-PrBCM by the longitudinal muscle at 30° C is shown in Figure 3a. Under these conditions a high proportion of the uptake is suppressed by 10^{-7} M atropine. The atropine-sensitive uptake (Fig. 3b) is virtually complete at 2 h and reaches a value of 145 pmol/g wet weight. This compares with a value of 125 pmol/g for the uptake of BCM sensitive to 3×10^{-8} M atropine (Rang, 1967) and a value of 180 pmol/g for the high affinity binding site for atropine (Paton & Rang, 1965). If the aziridinium ion was first hydrolysed by allowing the solution to stand at room temperature for four days, the uptake after 90 min incubation with the strips was negligible (4 ± 1 pmol/g). The rate constant for formation of the reversible [^{3}H]-PrBCM-receptor complex, assuming the same kinetic scheme as for antagonism of the contractile response (Gill & Rang, 1966), was 1.4×10^5 $M^{-1} s^{-1}$, which agrees reasonably well with the value obtained from antagonism of the contractile response (1.7×10^5 $M^{-1} s^{-1}$, Young *et al.*, 1972) but contrasts with the higher value to be expected from the 1.2×10^6 $M^{-1} s^{-1}$ obtained by Gill & Rang (1966) with BCM at 37° C.

The inhibition of [^{3}H]-PrBCM binding as a function of atropine concentration is shown in Fig. 4, where it can be seen that 10^{-7} M atropine produced about the maximum degree of inhibition of uptake obtainable. Half-maximal inhibition required about 7×10^{-9} M atropine, which does not agree well with the result expected from an antagonist with a single binding site of affinity

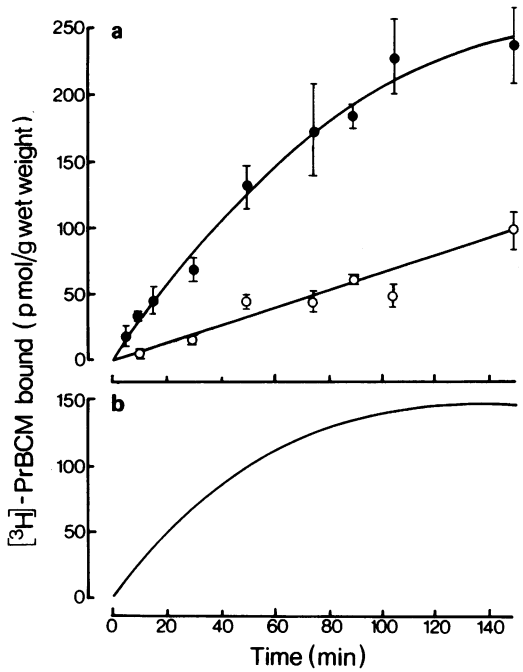

Fig. 3 (a) Time course of the binding of 2.4 nM [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) by longitudinal muscle strips at 30° C. Binding was measured as described in the methods section either without further drug addition (•), or in the presence of 100 nM atropine (○). Each point is the mean of 4-11 determinations on strips derived from at least two guinea-pigs. The vertical bars indicate ±s.e mean. (b) Time course of the atropine sensitive uptake.

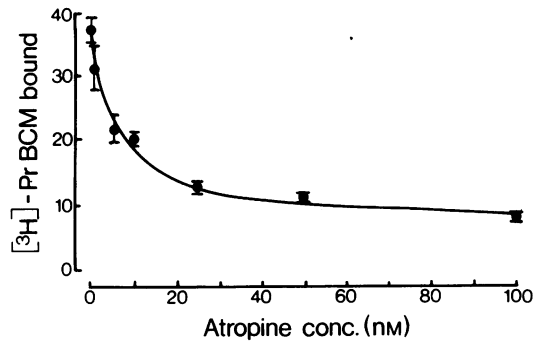

Fig. 4 Inhibition of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) by atropine. Incubation with [^{3}H]-PrBCM was for 10 min at 30° C. Each point is the mean of 5-17 determinations except that for 10^{-8} M atropine which was derived from 27 strips. For some of the determinations the strips were first exposed to 10^{-6} M atropine for 30 min, followed by extensive washing against the concentration of atropine to be tested. The vertical bars indicate ±s.e. mean.

near 10^9 M^{-1}. Results with other antagonists and with the cholinesterase inhibitors, neostigmine and physostigmine, are given in Table 1. At these concentrations only the antimuscarinic drugs, atropine and isopropamide, significantly affected the binding of [^{3}H]-PrBCM.

At higher concentrations the agonists, acetylcholine, carbachol, methylfurmethide and hexyltrimethylammonium, also inhibited uptake, the maximum inhibition obtained being approximately the same as that obtained with atropine. The concentrations producing a 50% inhibition of the drug-sensitive uptake are given in Table 2.

Effect of exposure of muscle strips to distilled water on uptake of [^{3}H]-propylbenzilylcholine mustard

Exposure of muscle strips to distilled water for 30 min before the normal preincubation in Krebs

Table 1 Effect of competitors on uptake of propylbenzilylcholine mustard (PrBCM).

Drug	*Conc. (M)*	*Uptake time (min)*	*% inhibition*	*% inhibition by atropine*	*No.*
Atropine	10^{-7}	10	*78 ± 3		17
Hexamethonium	10^{-7}	10	−3 ± 18		5
(+)-Tubocurarine	10^{-6}	10	−13 ± 13	*74 ± 3	4
Mepyramine	10^{-7}	10	−6 ± 21		5
Neostigmine	10^{-7}	10	19 ± 10		5
,,	10^{-7}	90	7 ± 8	*68 ± 4	6
Physostigmine	10^{-7}	90	1 ± 14	*68 ± 4	6
Decamethonium	10^{-7}	90	3 ± 16	*62 ± 7	9
Isopropamide	10^{-7}	90	*62 ± 6	*60 ± 8	6

Incubation with 2.4 nM [^{3}H]-PrBCM at 30°C. * $P < 0.001$.

Table 2 Inhibition of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) binding by muscarinic agonists.

Agonist	*C½ (M)*
Acetylcholine*	9×10^{-6}
Carbachol	8.3×10^{-5}
Hexyltrimethylammonium	3.3×10^{-5}
Methylfurmethide	8.3×10^{-6}

* Neostigmine (10^{-7} M) present. C½ is the agonist concentration giving 50% inhibition of the drug sensitive uptake after 10 min incubation with [^{3}H]-PrBCM. In all cases, the non-specific binding was taken as 18% of the control uptake.

Table 4 Effect of temperature on the binding of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM).

Temperature	*Atropine-sensitive uptake (pmol/g*)*	*Atropine-insensitive uptake (pmol/g*)*	*Atropine-sensitive uptake (% of total)*
10°C	17.3	1.7	91
20°C	20.0	3.0	87
30°C	24.5	6.5	79
Activation energy (kcal/mol)	3.0	11.5	

* After incubation for 10 min with 2.4 nM [^{3}H]-PrBCM.

solution and exposure to [^{3}H]-PrBCM, resulted in a large increase in both atropine-sensitive and atropine-resistant uptake (Table 3). This was not due to loss of weight since, in treated strips, this was only 7% less than in paired controls. The overall effect of distilled water was to increase the apparent concentration of atropine-sensitive sites, as deduced from the 150 min incubation, from 145 pmol/g to 330 pmol/g. However, the proportion of the total uptake of [^{3}H]-PrBCM sensitive to atropine was the same in the treated as in the untreated strips, as shown by the more extensive series of experiments with a 10 min incubation period (Table 3).

Effect of temperature

The rate constant for the atropine-sensitive uptake of [^{3}H]-PrBCM had a low temperature dependence ($E_a \sim 3$ kcal/mol) (Table 4). The measurements at 10° and 20°C are based on a limited number of points and consequently the values for k_1 cannot be considered as more than approximations. However, it is clear that the temperature-dependence of the atropine-sensitive uptake is much less than that of the atropine-insensitive binding ($E_a = 11.5$ kcal/mol), a pseudo-zero order process, where the rate was approximately halved by each 10°C fall in temperature. The consequence of the differential temperature-dependence is that, as the temperature is lowered, the proportion of the total uptake that is atropine-sensitive increases (Table 4), such that at 10°C, after a 10 min exposure to [^{3}H]-PrBCM, 91% of the uptake was atropine-sensitive.

Relation between rate of loss of bound tritium and recovery from block

To test the hypothesis that the slow recovery of the contractile response after block by PrBCM depends on the hydrolysis of the covalent bond between the antagonist and the receptor, the rate of recovery was compared with the rate of loss of tritium from the tissue.

To determine the rate of recovery of the

Table 3 Effect of pre-exposure of muscle strips to distilled water on the uptake of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM.

Pretreatment	*Incubation time (min)*	*[^{3}H]-PrBCM bound (pmol/g wet weight)*			*Atropine-sensitive uptake (% of total)†*
		Total	*Atropine-sensitive**	*Atropine-resistant*	
Krebs 30 min	150	239 ± 39 (5)	166 ± 39	74 ± 8 (5)	
Water 30 min	150	563 ± 44 (5)	331 ± 56	232 ± 35 (5)	
Krebs 30 min	10	36 ± 2 (17)	28 ± 2	8 ± 1 (17)	78 (74-82)
Water 30 min	10	109 ± 10 (16)	82 ± 10	27 ± 2 (16)	75 (67-80)

* Atropine concentration 10^{-7} M. † With 95% confidence limits. After the pretreatment all strips were incubated in Krebs solution for 1 h before the addition of [^{3}H]-PrBCM. Values are means ± s.e. (number of strips).

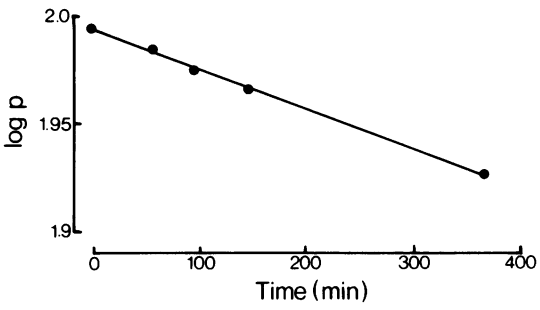

Fig. 5 Rate of recovery of the contractile response in muscle strips treated with propylbenzilylcholine mustard. The percentage of receptors occluded by the antagonist, p, was obtained from the relationship: p = (dose ratio − 1)/(dose-ratio) (Paton, 1961). Temperature, 37°C. Agonist, Carbachol.

contractile response, strips were incubated with PrBCM until a dose-ratio of approximately 100 was achieved. The antagonist was then washed out and dose-response curves obtained at intervals for about 6 hours. The fraction of receptors occluded by the antagonist declined exponentially (Fig. 5), consistent with a pseudo-first order hydrolysis. The rate of recovery showed a marked temperature-dependence (Table 5), making measurements at 30°C very difficult.

The rate of loss of receptor-bound tritium was determined from the difference, before and after a period of washing, between strips treated with [^{3}H]-PrBCM in the presence of 10^{-7}M atropine and strips labelled in the absence of atropine. In this case we have not established that the process is first order, since very long periods of washing were required, even at 37°C, to obtain a decline in the amount of tritium much greater than experimental error, making it difficult to obtain an adequate spread of experimental points. First order decay has been assumed and the rate constants obtained are set out in Table 5.

The agreement between the two sets of values is good and supports the hypothesis that recovery of the contractile response depends on the rate of removal of the antagonists from the receptor.

Table 5 Recovery of activity and loss of tritium on extended washing.

	37°C	*30°C*
Recovery of receptor availability	2×10^{-6} (1) s^{-1}	$6.6 \pm 2.0 \times 10^{-6}$ (4) s^{-1}
Loss of atropine sensitive bound tritium	1×10^{-6} (2) s^{-1}	$6.2 \pm 1.4 \times 10^{-6}$ (3) s^{-1}

Discussion

The synthesis of [^{3}H]-PrBCM described above provides a convenient route to a compound of high specific radioactivity coupled with high radiochemical purity. For this reason we have examined the properties of [^{3}H]-PrBCM, as an alternative to BCM, as an affinity label for the muscarinic receptor; the experiments reported here demonstrate that these are in general satisfactory. Under appropriate conditions a high proportion of the uptake of PrBCM is atropine-sensitive and can be followed to completion. The finding that distilled water increases the sites available for alkylation parallels situations where damage to membrane structure exposes binding sites for a variety of macromolecules (Wallach, 1972; Cuatrecasas, 1971) has shown that the number of insulin receptors in fat cells is apparently increased three- or four-fold by agents which disturb membrane phospholipids. In the present case, however, it is notable that the proportion of the total uptake that is atropine-sensitive is the same before and after distilled water treatment, and it may well be that the primary effect of the treatment is to cause complete relaxation of the strips thereby increasing the total membrane surface accessible to the medium. The maximum concentration of receptors found is 330 pmol/g, corresponding to about 10^6 molecules per cell or 3×10^{10} molecules/cm^2 cell surface.

The relatively low potency of atropine in inhibiting [^{3}H]-PrBCM binding is not readily explicable, and the half-inhibitory concentration does not agree with the affinity derived from experiments in which it is measured by antagonism of agonist action on contraction or ion flux. While there are a number of possible explanations of this discrepancy, it seems most likely that it is a property of the tissue rather than of the affinity label or the kinetic situation since consistent curves have been obtained with receptors in brain synaptosomes (Burgen, Hiley & Young, unpublished observations) and chick amnion (Cuthbert & Young, 1973). The failure of the kinetics of atropine uptake to satisfy a mass action equation for a simple receptor species (Paton & Rang, 1965) may represent another aspect of the same problem.

The inhibition of the uptake of an affinity label has not previously been utilized as a method for obtaining the affinity constants of muscarinic

agonists. Providing that the uptake of [^{3}H]-PrBCM at the end of the exposure is a small proportion of the saturating uptake, as should be the case with a 10 min incubation, then the concentration of agonist for 50% inhibition should be a good estimate of the binding affinity. However, the significance of the apparent affinity constants obtained must be considered against the strong probability that the agent is transforming the receptor into one with altered binding properties and that the overall effect in blocking [^{3}H]-PrBCM binding may be due to a combination of occupied and transformed receptors. This complexity may be one reason why numerical agreements with affinity constants derived from other approaches is not always satisfactory. There is, for instance, little agreement with the values determined by Burgen & Spero (1968) against contractile or flux responses or with the value for acetylcholine (9.3×10^5 M^{-1}) obtained by Sastry & Cheng (1972). It is, however, of interest that the apparent binding constant for the one partial agonist examined, hexyltrimethylammonium 3×10^4 M^{-1}, is in good agreement with the values obtained in several laboratories and from various approaches (summarized by Parker, 1972).

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