Br. J. Pharmac. (1974), **51**, 279-285

THE PROPERTIES OF MUSCARINIC RECEPTORS IN MAMMALIAN CEREBRAL CORTEX

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1 The reaction of tritiated propylbenzilylcholine mustard ([^{3}H]-PrBCM; *N*-2′-chloroethyl-*N*-[2″,3″-$^3H_2$]-propyl-2-aminoethylbenzilate) with homogenates of mammalian brain has been studied.

2 The uptake can be divided into an atropine-sensitive component of fixed capacity (380 pmol/g protein in the rat) and an atropine-insensitive part.

3 The atropine-sensitive portion is identified as muscarinic receptor by its insensitivity to nicotinic antagonists and anticholinesterases and its sensitivity to a range of muscarinic antagonists.

4 The uptake of [^{3}H]-PrBCM is also inhibited by muscarinic agonists and there is reasonable quantitative agreement between the affinities of agonists estimated in this way and in intact tissues by physiological responses.

5 The fraction of [^{3}H]-PrBCM uptake inhibited by muscarinic antagonists and agonists is the same.

6 The amount of receptor found in six mammalian species was inversely related to the size of the brain, but the rates of alkylation and the sensitivity to atropine were not dissimilar.

Introduction

The evidence that acetylcholine receptors of the muscarinic type are present in brain is convincing. At a number of cellular loci responses to muscarinic agonists have been demonstrated and these are antagonized by atropine and other antimuscarinic drugs: these data have been summarized by Curtis & Crawford (1969). On the other hand little quantitative work has been undertaken on these receptors and it is not known how far their characteristics are similar to or differ from those found in better studied systems in peripheral tissues.

The objective of this paper is to define quantitatively the properties of the muscarinic receptors in cerebral cortex using the uptake of the tritium labelled alkylating muscarinic antagonist, propylbenzilylcholine mustard ([^{3}H]-PrBCM).

Methods

Preparations from mice, rats, and guinea-pigs were from decapitated animals; dogs were killed by intravenous pentobarbitone, and monkeys by intravenous phencyclidine. Pigs' heads were obtained from the slaughter house within a few hours of death.

After removal of the pia mater, the cortical grey matter was carefully separated and homogenized in 10 volumes of cold 320 mM sucrose solution in a Potter-Elvehjem homogenizer. After determination of the protein concentration by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as a standard, the homogenate was diluted in Krebs-Henseleit solution (equilibrated with 5% CO_2 in oxygen) to give a concentration of 0.1-0.27 mg protein/ml. When synaptosomes were to be prepared, the homogenate was centrifuged at 2000 *g* for 5 min, the supernatant removed and centrifuged at 17,000 *g* for 30 minutes. The pellet was then resuspended in its own volume of 320 mM sucrose and 3 ml of the suspension was layered on to 8 ml of 800 mM sucrose which overlay 8 ml of 1200 mM sucrose. The tube was then spun at 100,000 *g* for 1 hour. The synaptosome fraction was located at the interface between the 800 and 1200 mM sucrose layers.

The aziridinium of *N*-2′-chloroethyl-*N*-[2″,3″-

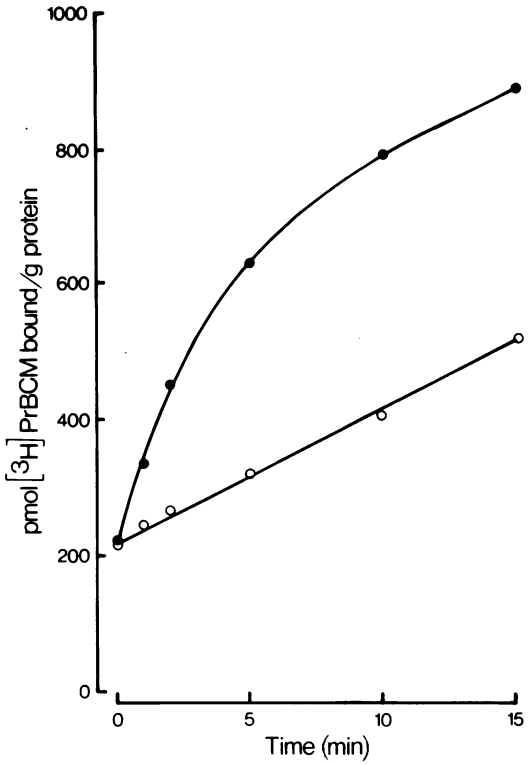

Fig. 1. , Uptake of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) by rat cortical synaptosomes. Synaptosome concentration: 0.27 mg protein/ml; [^{3}H]-PrBCM (2.4×10^{-9} M). (•) uptake in absence of atropine; (○) uptake in presence of atropine (10^{-7} M) added 15 min before the aziridinium. The points represent triplicate determinations. The s.e. is within the size of the points.

$^3H_2$]propyl-2-aminoethylbenzilate was prepared as described by Burgen, Hiley & Young (1974) and is referred to in the text as [^{3}H]-PrBCM. In most experiments the [^{3}H]-PrBCM was added to 3 ml brain homogenate and the incubation conducted at 30° C. The reaction was stopped by the addition of 20 ml cold Krebs-Henseleit containing 10 mM sodium thiosulphate. The suspension was filtered through a Millipore HAWP 02500 filter, which was then washed with 30 ml Krebs-Henseleit solution.

The filter was placed in a scintillation vial with 15 ml 0.4% Butyl PBD (Ciba, Duxford) dissolved in Triton X-100 : toluene : water (4 : 8 : 1) and counted in a Packard Tricarb scintillation counter. Activity was expressed as d/min after correction for efficiency and quenching with a standard curve. Where the effects of inhibitors of the uptake was being measured, the brain suspension

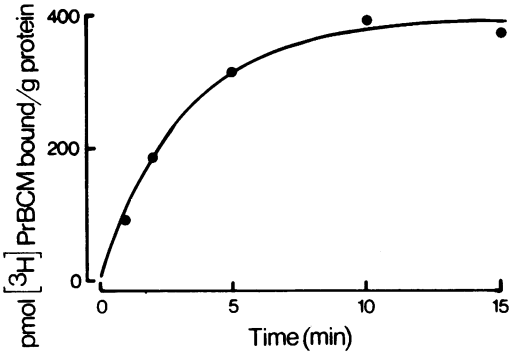

Fig. 2 Time curve of atropine-sensitive uptake from data of Figure 1. Curve is calculated from U_∞ = 390 pmol/g protein. $k_2 = 2.3 \times 10^6$ l mol^{-1} s^{-1}.

was normally incubated with the inhibitor for 15 min before the addition of the [^{3}H]-PrBCM.

Results

Figure 1 shows the time course of uptake of [^{3}H]-PrBCM (initial concentration 2.4×10^{-9} M) by a synaptosome preparation from rat cerebral cortex. It can be seen that 210 pmol/g protein has been bound to the tissue in a sample taken immediately after mixing, thereafter the uptake increased with time and reached a total of 890 pmol/g protein after 15 minutes. In the presence of 10^{-7} M atropine the initial uptake was the same, but the increase with time was much smaller and the total uptake after 15 min was only 520 pmol/g protein. When the atropine-sensitive part of the labelling is plotted (Fig. 2) it can be seen to be virtually complete after 10 minutes. The continuous line in this figure is a fitted exponential curve of the form:

$$U_t = U_\infty [1 - \exp(-Mk_2 t)]$$

where M is the concentration of alkylator; k_2 the rate constant for alkylation and U is uptake.

For the data of this experiment the uptake at infinite time (U_∞) was 390 pmol/g protein and the rate constant for the reaction (k_2) was 2.3×10^6 l mol^{-1} s^{-1}. The constant k_2 is the overall reaction constant of [^{3}H]-PrBCM for the receptor.

When the cortex preparations were pre-incubated for periods longer than 40-60 min a slow fall off in receptor concentration was seen; the receptor in this form is evidently rather thermolabile. On the other hand brain homogenates could be stored at 4° C for at least a week without significant loss of activity.

Specificity of inhibition by antagonists

It can be seen in Table 1 that the uptake of [^{3}H]-PrBCM by rat brain homogenates was not significantly changed by (+)-tubocurarine, hexamethonium, decamethonium, or α-bungarotoxin in concentrations that would be expected to cause a high level of occupancy of nicotinic receptors. It was also unaffected by neostigmine or physostigmine in concentrations adequate to give nearly total inhibition of acetylcholinesterase. On the other hand, hyoscine, methylatropinium, benzhexol, isopropamide, and caramiphen, all well-established muscarinic antagonists, were able to inhibit uptake of [^{3}H]-PrBCM by an amount similar to that for atropine.

Quantitative measurement of inhibition by atropine

It was pointed out earlier that the specific uptake of [^{3}H]-PrBCM can be accurately described by an exponential curve as would be expected for a first order reaction in which the concentration of the reactant, i.e. the [^{3}H]-PrBCM, remains substantially unchanged during the course of the uptake. In practice, since the initial amount of [^{3}H]-PrBCM is in ~10-fold excess of that taken up, and the rate of hydrolysis of aziridinium is small (Young, Hiley & Burgen, 1972), the deviation from strict first order conditions is acceptably small.

Table 1 Effect of various compounds on uptake of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) by rat cerebral cortex synaptosomes

	Concentration (M)	*Change in uptake (%)*
(+)-Tubocurarine	10^{-5}	+3
Hexamethonium	10^{-5}	0
Decamethonium	10^{-5}	−2
α-Bungarotoxin	1.9×10^{-6}	−1
Physostigmine	10^{-6}	+3
Neostigmine	10^{-5}	−6
Atropine	10^{-7}	−69
Hyoscine	10^{-8}	−69
Methylatropinium	10^{-8}	−64
Methylatropinium	10^{-7}	−67
Benzhexol	10^{-7}	−67
Caramiphen	10^{-7}	−61
Isopropamide	10^{-8}	−61
Isopropamide	10^{-7}	−73

Synaptosomes (0.27 mg protein/ml) were preincubated with the test compounds for 15 min before the addition of [^{3}H]-PrBCM, 2.4×10^{-9} M. The reaction was stopped 8 min later. All incubations were carried out in quadruplicate.

For measurements of inhibition of uptake two methods are open to us in principle. Firstly, we can allow uptake until only a small fraction of the receptor is alkylated; under these conditions the rate of uptake is quasi-linear with time and the equilibrium between a reversible inhibitor and the receptor is given directly by the degree of inhibition of uptake. To avoid significant errors the uptake must not exceed 15-20% of capacity. This method is perfectly acceptable in smooth muscle (Burgen *et al.*, 1974) where the receptor concentration is high and the atropine-insensitive uptake small. However, in the brain where the receptor concentration is lower and the atropine-insensitive uptake relatively high, this is not a very satisfactory method.

The alternative is to allow the alkylation of the receptors to proceed to 70-90% of completion in the control and to compute the rate constants for uptake. In these conditions the equilibrium with the reversible inhibitor is seen in the reduction of the rate constant for alkylation.

Thus in the control, uptake is:

$$U_t = U_\infty [1 - \exp(-Mk_2 t)]$$

and in the presence of the reversible inhibitor:

$$U'_t = U_\infty \left[1 - \exp\left(\frac{-Mk_2 t}{1+AK}\right)\right]$$

where A is the concentration and K the equilibrium affinity constant of the reversible antagonist.

It is unnecessary to extract the rate constants explicitly since the percentage reduction of the rate constant (I) is given by

$$I = \frac{100}{Mk_2 t} \ln\left(\frac{U_\infty - U'_t}{U_\infty - U_t}\right)$$

Figure 3 shows the time course of uptake of [^{3}H]-PrBCM in the absence and presence of 10^{-9} M atropine. It can be seen that calculation from the rate constants in the time taken to reach a given degree of uptake leads to a constant value for inhibition, whereas calculations based on direct use of the percentage uptake give an apparently decreasing inhibition if calculated at later time.

Now this analysis is based on the assumption that the system consisting of the reversible inhibitor and non-alkylated receptor sites has reached equilibrium in the preliminary incubation and remains in equilibrium during the alkylation of the receptors. While this is very likely to be the case with the weaker inhibitors, it is not necessarily going to be true for those antagonists which are known to equilibrate slowly such as

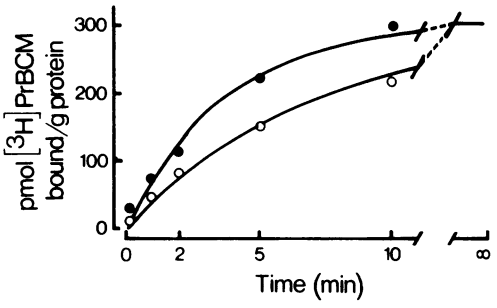

Fig. 3 Uptake of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) in the presence of atropine. The concentration of rat cortex homogenate was 0.27 mg protein/ml. After 15 min preincubation [^{3}H]-PrBCM was added to give a concentration of 1.93×10^{-9} M. (•) shows uptake in the absence of atropine after subtraction of non-specific uptake. (○) shows uptake in the presence of 1×10^{-9} M atropine. The capacity of the receptors in the preparation was 305 pmol/g protein. The solid curves are fitted to the equation $U_t = 305\ [1 - \exp(-Mk_2 t)]$; for the control, $k_2 = 2.3 \times 10^6$ l mol^{-1} s^{-1}, in the presence of atropine, $k_2 = 1.2 \times 10^6$ l mol^{-1} s^{-1} giving a value of $K = 1.1 \times 10^9$ M^{-1}.

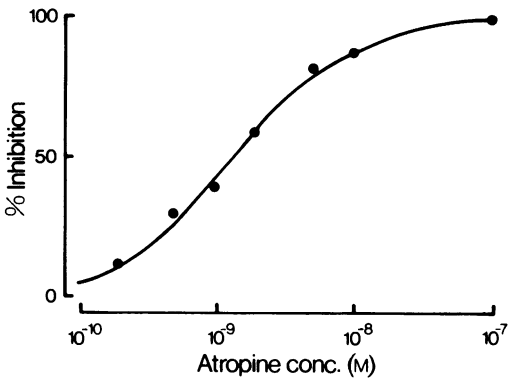

Fig. 4 Inhibition of uptake by atropine in the rat cortex. Homogenate containing 0.10 mg protein/ml was preincubated for 5 min with [^{3}H]-propylbenzilycholine mustard (1.2×10^{-9} M). The percentage inhibition was calculated from the change in rate constant of alkylation as described in the text.

atropine itself (Paton & Rang, 1965). The completeness of pre-equilibration therefore needs to be tested. We have done this by a comparison of pre-equilibration times of 5 min and 30 minutes. With atropine the difference was small, computer simulation showed it to be responsible for an error in the inhibitory constant of less than 20%.

In Fig. 4 the inhibition of uptake rate constant by atropine is shown as a function of atropine concentration. The continuous line is the best fit mass action equation with K = 8.0 (±0.5) $\times 10^8$ M^{-1}. Figure 5 shows a Hill plot of the data; the coefficient is 1.0 and thus gives no evidence of either positive or negative co-operativity in atropine binding.

Effect of muscarinic agonists on uptake

Similar inhibition experiments were carried out with agonists. Figure 6 shows that acetylcholine and pilocarpine both inhibit uptake, the maximum inhibition is not distinguishable experimentally from that produced by 10^{-7} M atropine and the experimental points fit a calculated mass action curve satisfactorily. Similar behaviour was found for carbachol, methylfurmethide and hexyltrimethylammonium. The inhibitory constants for these five compounds are shown in Table 2.

Subcellular fractionation

The results of a typical fractionation are shown in Table 3. Most of the receptor is in the pellet, and on subfractionation this is seen to be in the synaptosome fraction. Little enrichment of activity resulted from preparing synaptosomes and it is for this reason that most of the results reported here were carried out on simple homogenates.

Table 2 The effect of agonists on uptake of [^{3}H]-propylbenzilylcholine mustard ([^{3}H]-PrBCM) by rat cortex synaptosomes

	Inhibition constant M^{-1}	*Maximum inhibition*
Acetylcholine	4.0×10^5	99
Carbachol	4.8×10^4	103
Methylfurmethide	2.5×10^5	102
Pilocarpine	3.9×10^4	101
Hexyltrimethylammonium	8×10^4	99

Inhibitors were preincubated with synaptosomes (0.27 mg protein/ml) for 15 min and then [^{3}H]-PrBCM (2.4×10^{-9} M) added. The reaction was stopped after 8 minutes. In the case of acetylcholine, physostigmine (10^{-6} M) was present to inhibit cholinesterases. Maximum inhibition is expressed as a percentage of that in the presence of atropine (10^{-7} M).

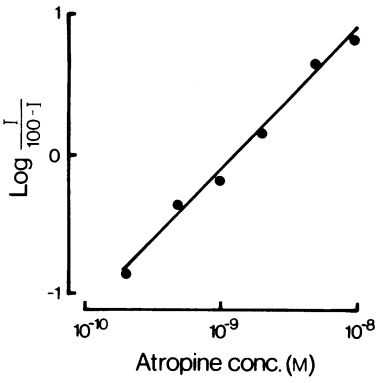

Fig. 5 Hill plot of data of Figure 4. Ordinates: log Inhibition (I)/100 – Inhibition (I). Slope = Hill coefficient = 1.0.

Receptors in the cerebral cortex of other animals

The concentrations of receptor, rates of alkylation, and atropine affinity constants for six mammalian species are listed in Table 4. It will be seen that there is an approximate inverse correlation of the amount of receptor with brain size of animal; a number of enzymes have been shown to follow this rule (Tower & Elliott, 1952). The rates of reaction and atropine inhibitory constants fall within a small range and suggest that the receptor in these species is very similar.

Discussion

The receptors in the brain delineated by atropine-sensitive uptake of [^{3}H]-PrBCM appear

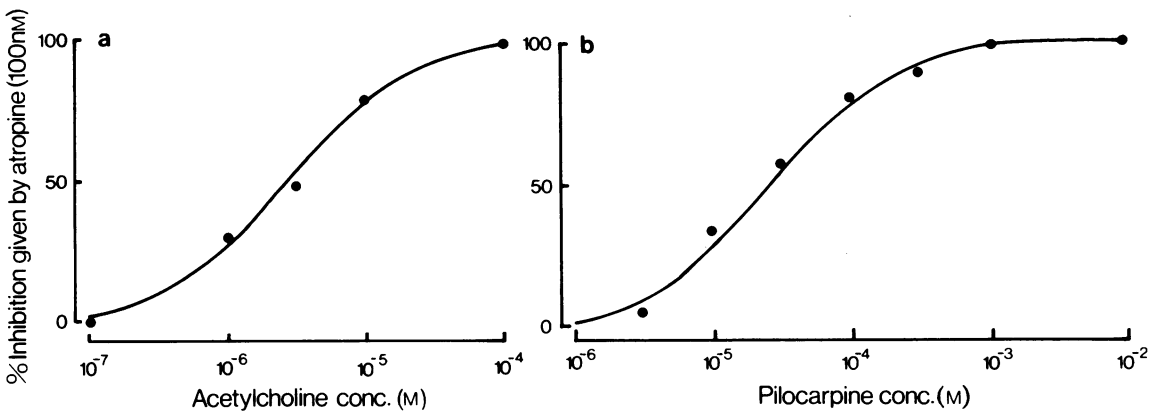

Fig. 6 Inhibition of uptake by (a) acetylcholine, (b) pilocarpine, solid lines are calculated mass action curves for (a) $K = 4.0 \times 10^5$ M^{-1} (b) $K = 3.9 \times 10^4$ M^{-1}.

Table 3 Subcellular fractionation of rat brain homogenates

	ml	*Protein (mg/ml)*	*Receptor (pmol/g protein)*	*Total receptor (pmol)*
Homogenate	30	9.4	366	102
17,000 *g* supernatant	30	2.8	293	25
17,000 *g* pellet (resuspended)	12.5	15.0	377	70
Myelin fraction (320 mM/800 mM interface)	20	2.1	195	8
Synaptosomes (800 mM/1200 mM interface)	29	3.3	434	42
100,000 *g* pellet (resuspended)	9.6	1.9	109	2

Receptor concentration was determined as being the difference between uptake after 15 min in samples (0.27 mg protein/ml) incubated with [^{3}H]-PrBCM (2.4×10^{-9} M) in the presence and absence of atropine (10^{-7} M). All samples were preincubated for 15 min before addition of the label.

to meet the criteria for a muscarinic receptor. The specificity shown by the lack of sensitivity to substances reacting with nicotinic receptors (including α-bungarotoxin), the sensitivity to six anti-muscarinic drugs as well as the sensitivity to muscarinic agonists all point in this direction. On the quantitative side the affinity for atropine in guinea-pig cortex found by us (1.0×10^9 M^{-1}) is in excellent agreement with the value of 0.9×10^9 M^{-1} found by antagonism of acetylcholine in the guinea-pig ileum by Paton & Rang (1965). Farrow & O'Brien (1973) have measured binding of atropine directly to homogenates of rat whole brain and found a two component binding process. The higher affinity process had a binding constant of 1.7×10^9 M^{-1} which is not very different from the constants of 0.8×10^9 M^{-1} and 1.1×10^9 M^{-1} found here. The capacity found by these authors was 89 pmol/g wet weight which is equivalent to about 800 pmol/g protein (rat brain contains ~0.11 g protein/g wet weight); this is twice the value found here for the cortex and the difference may be due to inclusion of higher activity areas.

The inhibitory curves for the agonists fit a simple mass action curve very well with a Hill coefficient close to unity. There is nothing to indicate a co-operative effect or the modified alkylation rate that would be expected if a metaphilic reaction were present (Rang & Ritter, 1969). The actual presumptive affinity constants agree rather well with the values obtained by Furchgott & Bursztyn (1967) with their method using dibenamine for carbachol (6.3×10^4 M^{-1}), acetylcholine (4.8×10^5 M^{-1}) and pilocarpine (1.4×10^4 M^{-1}) and by Parker (1972) for hexyltrimethylammonium (3.6×10^4 M^{-1}). These quantitative agreements reinforce the conclusion that our measurements concern a functional receptor which within the errors of the current experimental methods is a single entity. Of course it cannot be assumed that when we measured inhibitory potency of an agonist this necessarily has the same significance as in the case of an antagonist. All we are measuring is the failure to react with an alkylator which may indicate either competitive occupancy of the receptor site or alternatively that the agonist has converted the receptor to a form unable to react with the alkylator although not necessarily occupied by the agonist. The present experiments do not enable us to distinguish between these alternatives. Nevertheless the finding that the number of alkylation sites sensitive to agonists and antagonists is identical is important and suggests that the receptor sites for agonists and antagonists are either common or in a strict one to one stoichiometry.

In the six mammalian species examined the properties of the receptor showed great similarities, any structural differences in these receptors are likely to be quite minor. The quantitative agreement with the properties of muscarinic receptors in smooth muscle referred to earlier also suggest that the muscarinic receptors in the periphery and brain are probably identical in structure. However, the concentration of receptors in strips of guinea-pig longitudinal muscle was ~700 pmol/g protein (Burgen *et al.*, 1974) which is almost twice as high as in guinea-pig cortex.

Table 4 Comparison of receptor in cerebral cortex from six mammalian species

	Receptor concentrations (pmol/g protein)*	*Reaction rate constant for [^{3}H]-PrBCM* (l mol^{-1} s^{-1})*	*Atropine inhibitory constant** (M^{-1})*
Mouse	340	1.4×10^6	$1.2 \pm 0.1 \times 10^9$
Rat	370	2.3×10^6	$0.8 \pm 0.05 \times 10^9$
Guinea-pig	200	2.5×10^6	$1.0 \pm 0.4 \times 10^9$
Dog	200	3.2×10^6	$1.0 \pm 0.1 \times 10^9$
Pig	140	2.0×10^6	–
Macaque	110	2.1×10^6	$2.3 \pm 0.4 \times 10^9$

* Reaction rate constants and capacities were derived by following the time course of the reaction between cortical homogenates and [^{3}H]-PrBCM both in the presence and in the absence of atropine (100 nM). Experimental conditions as in Figure 1.

** Homogenates containing 0.27 mg protein/ml were incubated with [^{3}H]-PrBCM (2.4 nM) for 8 min in the presence of several concentrations of atropine (10^{-10} M to 10^{-7} M). Values are derived from the slope of the plot of 1/inhibition of rate constant versus 1/atropine concentration. The percentage inhibition of rate constant was calculated as described in the text.

Isopropamide was the gift of Janssen Pharmaceutica and the α-bungarotoxin a gift from Dr H. Friedmann.

C.R.H. thanks the Medical Research Council for a Scholarship for Training in Research Methods.

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(Revised November 28, 1973)