TABLE 3. EFFECT OF DRUGS ON ADP/O RATIOS

Concentration of drug	Azapropazone	Phenylbutazone	Sodium salicylate
2×10^{-3} M	65·6 ± 3		
$1{\cdot}3 \times 10^{-3}$ M	73·0 ± 4		
$6{\cdot}6 \times 10^{-4}$ M	82·8 ± 2		71·0 ± 3
$3{\cdot}3 \times 10^{-4}$ M			82·0 ± 2
$1{\cdot}6 \times 10^{-4}$ M		70·0 ± 3	
$6{\cdot}6 \times 10^{-5}$ M		85·9 ± 1	

Results expressed as percentage of control values adjusted to 100 per cent.
Each result represents mean value ± S.D. of four experiments.

The biochemistry of azapropazone is similar to the two established anti-inflammatory drugs phenylbutazone and sodium salicylate in that it has stabilised albumin and uncoupled oxidative phosphorylation.

A large number of acidic non-steroidal drugs possess these properties. Both these properties have a theoretical importance in that they may reduce inflammation. However, further work is needed to link biochemical properties *in vitro* with pharmacological action *in vivo*.

Clearly, non-steroidal anti-inflammatory drugs possess a common set of biochemical properties which individually, or collectively, may be responsible for their anti-inflammatory action. Azapropazone possesses at least three properties of potential anti-inflammatory value. The relevance of these properties to the situation *in vivo* has yet to be determined.

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REFERENCES

1. G. WEISSMAN and J. T. DINGLE, *Exp. cell. Res.* **25,** 207 (1961).
2. Y. MIZUSHIMA and H. SUZUKI, *Archs. int. Pharmacodyn. Ther.* **157,** 115 (1965).
3. S. S. ADAMS and R. COBB, *Nature, Lond.* **181,** 773 (1958).
4. Y. MIZUSHIMA and T. NAKAGAWA, *Rheumatism* **22,** 16 (1966).
5. C. C. CLARK, R. WOLF, D. GRANGER and Z. TAYLOR, *J. Appl. Physiol.* **6,** 189 (1953).
6. R. B. CAPSTICK, *M.Sc. Thesis,* University of Bath (1970).

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Further differentiation of cholinergic receptors in leech muscle

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THE CHOLINERGIC receptors of leech (*Hirudo medicinalis*) dorsal muscle, while formally classifiable as nicotinic,[1,2] appear to possess a number of features that distinguish them from the nicotinic receptors at vertebrate skeletal neuromuscular junctions. Thus, Flacke and Yeoh[2,3] argued for different receptors in the leech dorsal muscle for monoquaternary (acetylcholine, carbachol and nicotine) and bisquaternary (succinyl-choline, decamethonium) agonist ligands on the basis of the relative

potencies and different maximum responses obtained with the two groups of agonists. They further showed that gallamine, which serves as a competitive antagonist to both groups of agonists, gave pA_2 values[4] that were uniform within each group but differed by one log unit between groups. Additionally, tubocurarine behaved as a normal competitive antagonist to the monoquaternary agonists but did not antagonize the bisquaternary agonists, although it did produce a tachyphylaxis to repeated administration that was specific for the bisquaternary ligands. Rang and Ritter[5] have similarly distinguished the two classes of receptors by the observation that the irreversibly acting decamethonium analog, decamethylene-1-(*N*-benzyl-2-chloroethylamino)-10-dimethylbenzylammonium chloride, antagonized responses to succinylcholine but was completely ineffective against carbachol-induced responses.

The above findings raise questions of substantial interest as to the organization of cholinergic receptors in the leech relative to the vertebrates. We have endeavored to analyze the situation further by studying the effects of three receptor probes on the mechanical responses induced by carbachol (CCh) and succinylcholine (SCh) in the leech muscle. The probes utilized were dithiothreitol (DTT), which eliminates or reduces the responsiveness of *Electrophorus electrophax*[6,7] and chick biventer cervicis[8] preparations to monoquaternary ligands, probably by reducing a disulfide bond at or adjacent to the recognition site of the receptor,[9] *p*-trimethylammonium benzenediazonium fluoborate (TDF), which irreversibly inactivates the electroplax[7] and frog rectus (D. H. Ross and D. J. Triggle, unpublished data) to CCh and decamethonium, and α-bungarotoxin (α-Bgt), a constituent of *Bungarus multicinctus* venom, which is an extremely potent and specific inactivator of nicotinic receptors in the electroplax and vertebrate skeletal neuromuscular junctions.[10–13]

We employed the leech dorsal muscle preparation as described by Perry,[14] and the results with these probes confirm the thesis that substantial differences exist in the topography or constitution (or both) of the receptors for CCh and SCh in the leech. Thus, DTT produces a small parallel shift to lower sensitivity with CCh, but an approximately 50 per cent depression of the response to SCh (Table 1). Both of these effects of DTT were fully reversed with the oxidizing agent, 5,5′-dithiobis (2-nitrobenzoic acid). Hexamethonium was ineffective as an agonist before and after DTT treatment, a situation in marked contrast to that with the electroplax[15] and chick biventer cervicis,[8] where DTT treatment converts the action of hexamethonium from feebly antagonistic to markedly stimulant. TDF produced a small decrease in sensitivity to CCh, but the same concentration reduced the maximum response to SCh approximately 50 per cent; the inhibitory effect of TDF on the SCh-induced response was potentiated by prior DTT treatment (Table 1). In the electroplax preparation, Podleski *et al.*[7] found that DTT treatment converted the actions of TDF from irreversible antagonism to reversible agonism. α-Bgt showed a very marked selectivity of action, being completely ineffective against CCh while virtually eliminating all response to SCh (Table 1). DTT pretreatment did not change the inactivity of α-Bgt toward CCh-induced responses (Table 1). The response to 100 mM K^+ was completely unaffected by α-Bgt, TDF and DTT at the concentrations and exposures shown in Table 1, indicating the selectivity of these antagonists for processes mediated through the cholinergic receptor.

Our results with these three probes confirm and extend previous observations[2,5] describing the marked differences between mono- and bis-quaternary ligand activity in the leech dorsal muscle. The effects of DTT and TDF are significantly more marked on SCh-induced responses and, of particular significance, α-Bgt is without effect on CCh-induced responses but eliminates completely SCh-induced responses. Since in vertebrate preparations[10–13] α-Bgt does not distinguish between the binding of mono- and bis-quaternary ligands, it is clear that there must exist significant differences in cholinergic receptor organization in the leech and vertebrates. The basis of these differences remains obscure, but there are several obvious possibilities.

There may exist entirely different receptors, either distinct proteins carried in the same or separate cells or distinct binding sites within the same protein, for mono- and bisquaternary ligands in the leech; in the vertebrates, a good case can be made that the binding sites for these ligands are partially overlapping[16–19] with the selective toxins showing no discrimination between mono- and bisquaternary ligands. In view of the absolute selectivity of α-Bgt (mol. wt. = 8000) in leech muscle for SCh, it appears simpler at the present time to visualize leech muscle mono- and bisquaternary receptors as carried on distinct proteins. Whether these are carried in the same or different cells is not known; however, there is good evidence from ganglion cells that different types of cholinergic receptors are present in a given cell.[20] That *d*-tubocurarine should inhibit CCh-induced responses[2,3] does not negate a concept of entirely distinct mono and bisquaternary binding sites, since recent structural determinations have revealed it to have a monoquaternary structure.[21]

In connection with this proposal, the work of Khromov-Borisov and Michelson[17] suggesting that bisquaternary sensitivity is a late evolutionary development seems of interest, since the leech is the lowest order showing sensitivity to the C_{10} bisquaternary structure and this, according to Michelson, may represent the development of oligomeric receptor assemblies. It is conceivable that in the leech we see the existence of both monomeric (sensitive only to monoquaternary ligands) and oligomeric

TABLE 1. EFFECTS OF DTT, TDF AND α-BGT ON RESPONSES OF LEECH DORSAL MUSCLE TO CCh AND SCh

Treatment*	CCh, ED_{50}($M \times 10^{-6}$ ±S.E.M.)
Control	1·7 ± 0·19 (6)
DTT, 10^{-3} M/30 min	4·2 ± 0·41 (6)†
DTT, 10^{-3} M/30 min + DTNB 10^{-3} M/30 min	1·6 ± 0·20 (3)‡
TDF, 5×10^{-5} M/30 min	2·7 ± 0·27 (4)§
α-Bgt, 10 μg/ml/60 min	2·3 ± 0·31 (4)‡
DTT 10^{-3} M/30 min + α-Bgt 10 μg/ml/60 min	4·1 ± 0·23 (3)†
	SCh, % of max. response $(ED)_{99}$ ±S.E.M.
Control	100
TDF, 5×10^{-5} M/30 min	57 ± 2 (7)
DTT, 10^{-3} M/30 min	39 ± 8 (4)
DTT, 10^{-3} M/30 min + TDF 5×10^{-5} M/30 min	0
α-Bgt, 10 μg/ml/60 min	5–10 (4)

* Dorsal muscle of leech was prepared as described by Perry.[14] Cumulative concentration–response curves for CCh were determined up to 80 per cent of estimated maximum response as judged by preliminary experiments. Maximum responses to CCh were determined at the end of the experiment to avoid prolonged desensitization. Doses of SCh which gave maximal contraction could be given at 45-min intervals without producing desensitization. Comparison of a maximal dose before and after treatment by antagonists was used to estimate potency toward SCh-induced responses. In experiments in which the tissue underwent treatment with DTT before incubation with various other antagonists, the DTT-treated muscle was washed for 10–15 min, then the second antagonist was added to the bath. Each value is the mean observation from tissues of the number of animals indicated in parentheses. See text for abbreviations.

† Significantly different from controls, P = 0·001.

‡ Not significantly different from controls.

§ Significantly different from controls, P = 0·05.

(sensitive only to bisquaternary ligands) receptor assemblies, showing differential sensitivity to DTT and TDF, but with only the oligomeric system having affinity for α-Bgt. Subsequent evolution may have generated the single receptor assembly characteristic of vertebrates having affinity for both mono and bisquaternary ligands and at which α-Bgt does not exhibit the selectivity of antagonism that we have described for the leech.

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REFERENCES

1. F. C. MacIntosh and W. L. M. Perry, in *Methods of medical research* (Ed. R. W. Gerard), Vol. 3, p. 78. Year Book Publishers, Chicago (1950).

2. W. FLACKE and T. S. YEOH, *Br. J. Pharmac. Chemother.* **33,** 154 (1968).
3. W. FLACKE and T. S. YEOH, *Br. J. Pharmac. Chemother.* **33,** 145 (1968).
4. O. ARUNLAKSHANA and H. O. SCHILD, *Br. J. Pharmac. Chemother.* **14,** 48 (1959).
5. H. P. RANG and J. M. RITTER, *Molec. Pharmac.* **5,** 394 (1969).
6. A. KARLIN and E. BARTELS, *Biochim. biophys. Acta* **126,** 525 (1966).
7. T. PODLESKI, J.-C. MEUNIER and J.-P. CHANGEUX, *Proc. natn. Acad. Sci. U.S.A.* **63,** 1239 (1969).
8. H. P. RANG and J. M. RITTER, *Molec. Pharmac.* **7,** 620 (1971).
9. A. KARLIN, J. PRIVES, W. DEAL and M. WINNIK, *J. molec. Biol.* **61,** 175 (1971).
10. C. C. CHANG and C. Y. LEE, *Mems Inst. Butantan, simp. int.* **33,** 555 (1966).
11. J.-P. CHANGEUX, M. KASAI and C. Y. LEE, *Proc. natn. Acad. Sci. U.S.A.* **67,** 1241 (1970).
12. R. MILEDI and L. T. POTTER, *Nature, Lond.* **233,** 599 (1971).
13. E. A. BARNARD, J. WIECKOWSKI and T. H. CHIU, *Nature, Lond.* **234,** 207 (1972).
14. W. L. M. PERRY, in *Pharmacological experiments on isolated preparations*, p. 50. Livingstone, Edinburgh (1968).
15. A. KARLIN and M. WINNIK, *Proc. natn. Acad. Sci. U.S.A.* **60,** 668 (1968).
16. R. B. BARLOW, *Introduction to chemical pharmacology*, p. 87. Methuen, London (1965).
17. N. V. KHROMOV-BORISOV and M. J. MICHELSON, *Pharmac. Rev.* **18,** 1051 (1966).
18. D. J. TRIGGLE, *Neurotransmitter-receptor interactions*, Ch. IV. Academic Press, London, New York (1971).
19. M. KASAI and J.-P. CHANGEUX, *J. mem. Biol.* **6,** 58 (1971).
20. R. J. WALKER and A. HEDGES, *Comp. Biochem. Physiol.* **23,** 977 (1968).
21. A. J. EVERETT, L. A. LOWE and S. WILKINSON, *Chem. Commun.* 1020 (1970).

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Effects of drugs on the phospholipid metabolism of the pineal body of rats

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MANY investigators have shown that cholinergic stimulation results in an increased incorporation of ^{32}P into phospholipids, particularly phosphatidyl inositol and phosphatidic acid, in a number of glandular and neural tissues.[1] In the salivary gland, which is controlled also by the sympathetic nervous system, epinephrine was reported to stimulate the incorporation of ^{32}P into phosphatidyl inositol.[2] The pineal body is also known to be innervated by the sympathetic nervous system,[3] and effects of norepinephrine and dibutyryl cyclic AMP on the metabolism of phospholipids have been reported.[4] The present paper describes the changes in the phospholipid metabolism of the pineal body of rats by several autonomic drugs *in vitro*.

Male Wistar rats were killed by decapitation between 1000 and 1300 hr. The pineal bodies were removed and immediately incubated under 95% O_2 + 5% CO_2 for 3 hr at 37° in 1 ml of sterilized Krebs–Ringer bicarbonate, pH 7·4, which contained 5·5 μmoles/ml of glucose and 10–100 μC/ml of ^{32}P-orthophosphate. All radioactivities were corrected to a specific activity of 10^5 cpm/μg of phosphorus in the medium. After the incubation, the tissues were weighed and the phospholipids were extracted with a mixture of 2:1 (v/v) chloroform–methanol. The phospholipids were separated two-dimensionally on a silicic acid-impregnated paper (Whatman SG 81) by the method of Wuthier[5] using solvents 1 and 3. The air-dried papers were stapled to Sakura medical X-ray film and the films were exposed for 3 days. The autoradiograms were superimposed over the chromatograms and the individual labeled phospholipids were circled. The individual spots were cut out and transferred to liquid scintillation counting vials. The samples were counted in a Nuclear Chicago 6725 liquid scintillation spectrometer. The identification of most of the phospholipids was accomplished by comparison with reference standards. Phosphatidyl choline was obtained from Tokyo Kasei Company and phosphatidyl inositol was bought from Applied Science Laboratories, Inc. The standard for phosphatidic acid was made from egg yolk lethicin by the chloroplast of carrots and was purified by silicic acid column chromatography.[6] The fraction eluted with 3% methanol in chloroform corresponded to the spot of phosphatidic acid on Wuthier's[5] chromatogram, although the identity of phosphatidic acid has been questioned.[7] Phosphatidyl glycerol was extracted from *Micrococcus lysodeikticus* and was purified by silicic acid column chromatography.[8] The fraction eluted by 15% methanol in chloroform was used as the standard. Phosphatidyl ethanolamine was located with ninhydrin and identified by