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DETERGENT-LIKE ACTION OF TETRAPHENYLBORATE ON PHOSPHOLIPID LABELLING IN GUINEA PIG CORTEX SUBFRACTIONS[1]

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Abstract—The addition of 2×10^{-4} M sodium tetraphenylborate to particulate preparations of guinea pig cerebral cortex increases labelling of phosphatidic acid from γ-[^{32}P]-ATP two- to four-fold. The effect was observed in all subcellular fractions studied (nuclear, synaptosomal, mitochondrial and microsomal) and is not blocked by the addition of atropine. Changes in phospholipid labelling similar to those induced by tetraphenylborate can be demonstrated with sodium dodecyl sulphate or sodium desoxycholate. It is suggested that tetraphenylborate stimulates lipid labelling by a detergent-like activation of diglyceride kinase.

TETRAPHENYLBORATE, first synthesized by WITTIG *et al.* (1949) and employed for the quantitative determination of potassium has received widespread attention largely because of a variety of actions, which in part, can be linked to its potassium-chelating capacity. TPB has been reported to dissociate cells (RAPPAPORT & HOWZE, 1966*a*), to swell mitochondria (HARRIS & LEONE, 1966), uncouple oxidative phosphorylation (UTSUMI & PACKER, 1967), prevent the light-induced uptake of monovalent cations by chloroplasts (HORTON & PACKER, 1967), inhibit ATPases and a number of other enzymes (STANBURY & WICKEN, 1969) and to inactivate AChE of erythrocyte membranes (HERZ *et al.*, 1971). The interest of our laboratories in this compound stemmed from reports that TPB duplicates some pharmacological actions of ACh at the neuromuscular junction (SEIFTER *et al.*, 1968) and the previous finding that it stimulates phospholipid labelling in particulate fractions of goldfish brain *in vitro* (HOLLANDER *et al.*, 1969, 1970) in a way that apparently resembled the stimulation of phospholipid labelling by ACh. Extending our studies on phospholipid labelling in guinea pig nerve endings in response to cholinergic agents (SCHACHT & AGRANOFF, 1972*a*; 1973), we investigated further the mode of action of TPB on phospholipid metabolism.

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Abbreviations used: PhA, phosphatidic acid (1,2-diacyl-*sn*-glycero-3-phosphate); PhIP, $PhIP_2$, phosphatidylinositol phosphate, -diphosphate; TPB, sodium tetraphenylborate; DOC, desoxycholate; SDS, sodium dodecyl sulphate.

MATERIALS AND METHODS

Preparation of fractions

Cerebral cortex from albino, male guinea pigs weighing approx. 300 g (Camm Research, Wayne, N.J.) was homogenized and subfractionated essentially as described previously (SCHACHT & AGRANOFF, 1972*a*). A 10 per cent homogenate in 0·32 M sucrose was centrifuged for 10 min at 1000 *g*. The pellet was washed and centrifuged again to yield the nuclear fraction. The combined supernatant fractions were centrifuged for 20 min at 13,000 *g* to yield a crude nerve ending-mitochondrial pellet and a supernatant fraction containing cytosol and microsomes. The nerve ending-mitochondrial pellet was suspended in 30 ml of 0·32 M sucrose, and was layered over three tubes of a discontinuous gradient consisting of 10 ml each of 0·8 and 1·2 M sucrose, then centrifuged in an SW25 rotor for 120 min at 62,000 *g*. Fractions collected have been characterized previously (SCHACHT & AGRANOFF, 1972*a*): a myelin-rich fraction over 0·8 M sucrose, a nerve ending-rich fraction over 1·2 M sucrose and a pellet, containing mitochondria. These fractions as well as the 13,000 *g* supernatant fraction were pelleted by centrifuging for 30 min at 100,000 *g*. Each pellet was suspended in 0·32 M sucrose. The 'particulate fraction' was prepared by centrifuging a 1000 *g* supernatant for 60 min at 100,000 *g*, and suspending the resulting pellet in 0·32 M sucrose. All fractions were frozen in a dry-ice-acetone bath and thawed three times.

Incubations were performed at 37°C in air with shaking in the following incubation medium (final concentrations): 80 mM Tris–HCl, pH 7·2; 8 mM $MgSO_4$; 1 mM ATP and 1 mg protein in a final vol. 0·5 ml. Incubations were terminated and lipids extracted with acidified chloroform–

TABLE 1. LABELLING OF PHOSPHOLIPIDS IN SUBCELLULAR FRACTIONS

Fraction	Total protein (mg)	PhA		PhIP		$PhIP_2$	
		control	+TPB	control	+TPB	control	+TPB
		d.p.m. ^{32}P in lipid					
Nuclear	91	2745	5368	4470	5513	1243	1206
Mitochondria	47	1794	4495	1834	2195	278	330
Nerve endings	61	3115	5744	5012	4919	562	686
Myelin	14	1578	2594	3417	2165	5190	4564
Microsomes	72	1426	2566	2698	2732	304	343

Fractions were prepared from two cortices. The fractions (1 mg protein) were incubated for 5 min with 8 μCi [^{32}P]ATP as described in 'Methods'.

methanol (HAJRA *et al.*, 1968), separated by TLC (HOLLANDER *et al.*, 1970), located by radioautography, scraped and counted by liquid scintillation spectrometry.

Protein was measured spectrophotometrically (LOWRY *et al.*, 1951). γ-[^{32}P]ATP was prepared enzymatically (GLYNN & CHAPPEL, 1964). Sodium TPB was purchased from Sigma (St. Louis, Mo.), SDS from Fisher Scientific (Fairlawn, N.J.) and sodium DOC from Mann Research (New York). Data presented are generally averages of at least duplicate incubations with variability less than 10 per cent.

RESULTS

Labelling of subcellular fractions

Fractions obtained by differential and density gradient fractionation were incubated in the presence and absence of 10^{-4} M TPB (Table 1). There was an approximately two-fold variability in the basal unstimulated labelling of PhA/mg of protein, with the nerve ending fraction always showing the highest activity of incorporation. The labelling of PhIP and $PhIP_2$, the only other significantly labelled lipids, showed greater variability among the subcellular fractions, but was highest in the myelin-rich fraction. The degree of stimulation of PhA labelling by TPB was similar in all fractions, about two to three-fold, and was variable for PhIP and $PhIP_2$.

Conditions of labelling

The stimulation of PhA labelling was largely independent of changes in the incubation conditions. Basal labelling was highest around pH 7; TPB stimulation showed a broad optimum range (6·1–7·6). Omission of magnesium from the medium results in reduced lipid labelling, but the percentage TPB stimulation was unaffected by omission or addition of Mg^{2+} up to 25 mM. Similarly, presence of Na^+ and Ca^{2+} (up to 20 mM) did not alter the TPB effect. K^+, as expected from its precipitation with TPB, could completely abolish the action of TPB. 50 mM NaF, added to inhibit ATPases, increased basal labelling of PhA two-fold but TPB stimulated labelling also to a similar extent.

TPB dose response

Stimulated labelling of PhA shows a sharp optimum around 10^{-4} M TPB (Fig. 1). Further studies identified a maximal stimulation at 2×10^{-4} M when the protein present was 1 mg (2 mg/ml). Increased labelling of PhIP and $PhIP_2$ is less pronounced than in PhA but

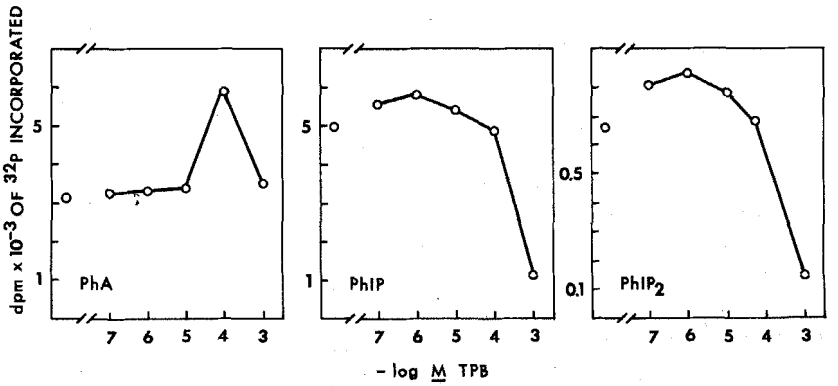

FIG. 1. The particulate fraction of guinea pig cerebral cortex (1 mg protein) was incubated for 5 min with 7 μCi [^{32}P]ATP as described in 'Methods'.

TABLE 2. INCUBATION OF TISSUE WITH TPB PRIOR TO LABELLING PULSE

Time of TPB addition	PhA	PhIP	$PhIP_2$
min	d.p.m. ^{32}P incorporated		
None added	942	2610	267
T_0	3406	3090	159
$-T_{10}$	3668	2871	131
$-T_{20}$	3669	2477	134
$-T_{30}$	3202	1573	105

The particulate fraction (1 mg protein) was pre-incubated for 30 min with 2×10^{-4} M TPB added at times indicated. T_{-30} indicates TPB was present for entire preincubation period. At T_0, ATP (2 μCi, 1 mM) was added and incubation continued for 5 min. Details as in 'Methods'.

clearly evident here and in comparable experiments (Tables 2, 4 and 5). Beyond the optimum concentration the lipid labelling is sharply decreased.

Miscellaneous

The effects of incubating the particulate fraction with TPB prior to the addition of isotope is shown in Table 2. Preincubation does not significantly increase PhA labelling. Extended treatment with TPB, however, lowers PhIP and $PhIP_2$ labelling. Other experiments were designed to study the effect of TPB on previously labelled lipids (Tables 3a and 3b) to observe possible degradative processes. Excess unlabelled ATP was added with TPB to lower its specific activity (Table 3a) or ATP was removed by reaction with deoxyglucose/hexokinase (Table 3b). There are no significant effects of TPB on the degradation of these lipids except for a suggestive decrease in PhIP in the presence of TPB (Table 3b).

Influence of atropine

To investigate the possible correlation between ACh- and TPB-stimulated phospholipid labelling, we studied the effect of atropine on the increased PhA labelling (Table 4a). Atropine did not block the TPB stimulation. Since this was in contrast to previous findings by HOLLANDER *et al.* (1970) in goldfish brain, we repeated these experiments (Table 4b). Here also atropine was ineffective in blocking the TPB-stimulated labelling in all of five separate experiments. The effectiveness of the atropine used was tested by employing the same preparation in experiments with guinea pig synaptosomes in which it effectively blocked ACh stimulation of PhA and PhI labelling (SCHACHT & AGRANOFF, 1973).

Comparison with known detergents

Since diglyceride kinase is known to be stimulated by certain detergents (HOKIN & HOKIN, 1959; LAPETINA & HAWTHORNE, 1971), we compared the effect of TPB on phospholipid labelling with the effects of some detergents in the same system. Sodium DOC, although stimulating PhA labelling to a far greater extent (Fig. 2), exhibited a similar pattern to TPB, a very narrow concentration optimum for PhA labelling, lesser stimulation of PhIP and $PhIP_2$ labelling and strong inhibition of labelling at higher concentrations. In addition, Table 5 shows that TPB in the presence of DOC does not produce any further stimulation (i.e. the two effects are not additive). SDS also showed a very similar effect on the incorporation of ^{32}P into the lipids (Fig. 3). To obtain an independent measure of detergency, we examined a dye-detergent interaction, with pinacyanol. Changes in

TABLE 3. EFFECT OF TPB ON PREVIOUSLY LABELLED LIPIDS

Treatment at		PhA	PhIP	$PhIP_2$
10 min	15 min	d.p.m. ^{32}P incorporated		
(a) Addition of unlabelled ATP[1]				
—	Incubation terminated	1529	2117	128
—	10 mM ATP	1436	1960	215
—	10 mM ATP + 2×10^{-4} M TPB	1537	2028	205
(b) Addition of deoxyglucose and hexokinase[2]				
Incubation terminated	—	2016	3011	356
Deoxyglucose/hexokinase	Incubation terminated	1826	3114	321
Deoxyglucose/hexokinase	None	1647	2718	212
Deoxyglucose/hexokinase	2×10^{-4} M TPB	1530	2382	194

[1] The particulate fraction (1 mg protein) was incubated for 15 min with 3 μCi [^{32}P]ATP. At this point the incubation was terminated or the additions indicated were made and incubations continued for further 10 min as described in 'Methods.'

[2] The particulate fraction (1 mg protein) was incubated for 10 min with 4 μCi [^{32}P]ATP as described in 'Methods'. At 10 min 2-deoxyglucose (35 mM) and hexokinase (Boehringer, Mannheim; 1 μl) were added when indicated; at 15 min further additions were made and remaining incubations were terminated 15 min thereafter.

TABLE 4. EFFECT OF ATROPINE ON TPB STIMULATION

Atropine	PhA		PhIP		$PhIP_2$	
	−TPB	+TPB	−TPB	+TPB	−TPB	+TPB
	d.p.m. ^{32}P incorporated					
(a) Guinea pig cortex[1]						
0	1960	7710	4480	5490	320	205
10^{-5} M	2119	7610	4701	5470	317	190
10^{-4} M	2190	7226	4532	5640	300	185
10^{-3} M	1940	6730	4531	5890	300	210
(b) Goldfish brain[2]						
0	3860	14550	1465	1460	150	110
10^{-4} M	3545	15080	1400	1435	175	130
10^{-3} M	3975	14330	1505	1470	173	110

[1] The particulate fraction (1 mg protein) was incubated for 5 min with 3 μCi [^{32}P]ATP. Atropine when indicated was added 15 min and TPB (2×10^{-4} M) was added 5 min prior to the addition of the isotope.

[2] Goldfish brain particulate fraction (0·9 mg protein) was incubated for 10 min with 10 μCi [^{32}P]ATP according to HOLLANDER *et al.* (1970). Atropine was added 10 min and TPB (5×10^{-4} M) was added 5 min prior to the addition of the [^{32}P]ATP.

absorbance have been interpreted to reflect micelle formation (ZAHLER *et al.*, 1968). An absorption minimum was obtained for both SDS and TPB in the 2–10 μg/ml range.

DISCUSSION

Increased labelling of phospholipids by $^{32}P_i$ in particular of PhA and phosphatidylinositol, in response to cholinergic agents has been well documented for brain tissue *in vivo* (SCHACHT & AGRANOFF, 1972*b*; FRIEDEL & SCHANBERG, 1972) as well as *in vitro* (HOKIN & HOKIN, 1958; YAGIHARA & HAWTHORNE, 1972; SCHACHT & AGRANOFF, 1972*a*). Suggestions have been made that this phenomenon is related to ion transport (HOKIN *et al.*, 1960), synaptic events (DURELL *et al.*, 1969), or neurotransmitter uptake (SCHACHT, NEALE & AGRANOFF, in press). Its precise nature, however, remains uncertain. Similarly, various explanations have been offered for the biochemical mechanism of the stimulated lipid labelling: stimulation of diglyceride kinase (HOKIN, 1969); stimulation of phosphodiesteratic cleavage of phosphatidylinositol (DURELL *et al.*, 1968) or PhIP and $PhIP_2$ (DURELL *et al.*, 1969). Recently, a stimulation of PhA hydrolysis by ACh has been shown (SCHACHT & AGRANOFF, 1973).

These biochemical investigations were hampered by the fact that the effect of ACh or carbamylcholine on CNS phospholipids could be observed only in whole tissue (brain slices) or intact subcellular fragments such as nerve ending particles. Although nerve endings may lend themselves more readily to studies of biochemical mechanisms than whole tissue, they are resealed anucleate neuronal fragments undoubtedly capable of

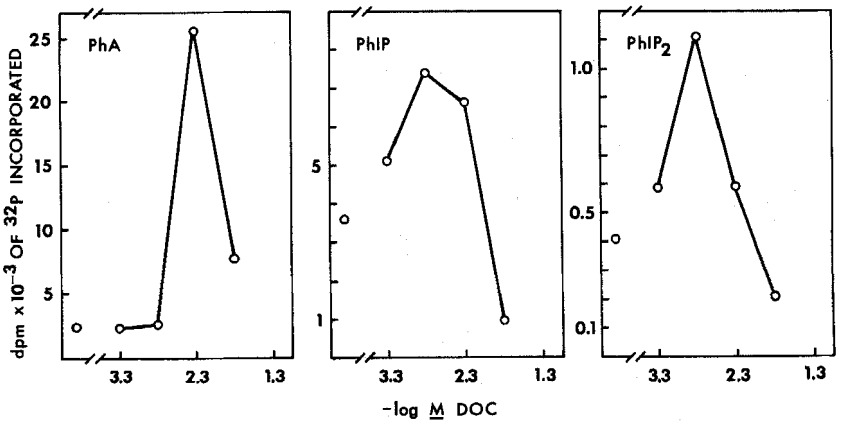

FIG. 2. The particulate fraction of guinea pig cerebral cortex (1 mg protein) was incubated for 5 min with 3 μCi [^{32}P]ATP as described in 'Methods'.

TABLE 5. INFLUENCE OF TPB AND DOC ON LIPID LABELLING

Desoxycholate	PhA		PhIP		$PhIP_2$	
	−TPB	+TPB	−TPB	+TPB	−TPB	+TPB
			d.p.m. ^{32}P incorporated			
0	2100	6400	3034	4100	353	325
$1{\cdot}5 \times 10^{-3}$ M	2630	6100	5995	3225	968	390
5×10^{-3} M	15950	12310	5205	2122	105	65

The particulate fraction (1 mg protein) was incubated for 5 min with 3 μCi [^{32}P]ATP as described in 'Methods'. TPB was 2×10^{-4} M.

compartmentalization present in whole cells. The report, therefore that TPB could act as a parasympathomimetic drug (SEIFTER *et al.*, 1968) and also stimulate phospholipid labelling in broken cell preparations of goldfish brain (HOLLANDER *et al.*, 1969, 1970) gave promise that TPB might serve as a probe in the elucidation of the biochemistry of the ACh effect.

In the present studies with guinea pig cortex subcellular fractions, TPB stimulated labelling of PhA from γ-[^{32}P]ATP approx. two- to three-fold in all subcellular fractions studied (Table 1). This contrasts with the effect of ACh on lipid labelling which we had shown to be specific for a 'light' and therefore presumably cholinergic nerve ending fraction (SCHACHT & AGRANOFF, 1972*a*). Labelling of PhIP and $PhIP_2$ was hardly affected at this concentration of TPB. The dose–response curve for TPB showed a sharp optimum for stimulated labelling around 10^{-4} M and a decrease in labelling of the polyphosphoinositides occurred at concentrations which were no longer stimulatory for PhA labelling. Thus, hydrolysis of PhIP and/or $PhIP_2$ does not appear to be directly linked to the stimulation of PhA labelling (HOLLANDER *et al.*, 1970). In other experiments with cholinergic agents the increase in PhA labelling and decrease in PhIP and $PhIP_2$ labelling have been dissociated on a number of grounds (SCHACHT & AGRANOFF, 1974). Further evidence against the possibility that formation of diglyceride *via* degradation of PhIP and/or $PhIP_2$ is responsible for increased PhA labelling by TPB comes from present experiments in which preparations were incubated with TPB prior to labelling (Table 2) or labelled prior to the addition of TPB (Table 3). Although pre-incubation with TPB lowers PhIP levels at longer times, it is not accompanied by labelling of PhA exceeding that achieved by adding tetraphenylborate at 0 time; there is, therefore, no evidence for the formation of an intermediate during the preincubation period, nor is the decrease in PhIP (Tables 2 and 3) of the order of magnitude that would suggest that this lipid serves as a diglyceride donor.

A block by atropine of the TPB effect, the strongest evidence for a possible cholinergic action of TPB on phospholipid labelling (HOLLANDER *et al.*, 1970), was not evident in incubations with guinea pig cortex fractions (Table 4). We therefore reinvestigated the TPB and atropine actions on goldfish brain particulate fractions (Table 4b). Here also, no block of TPB-stimulated labelling by atropine was observed.

The strikingly sharp concentration optimum for TPB-stimulated labelling of PhA in goldfish brain (HOLLANDER *et al.*, 1970) as well as in our studies (Fig. 1) suggested comparison to the stimulation of diglyceride kinase by DOC where a similar observation

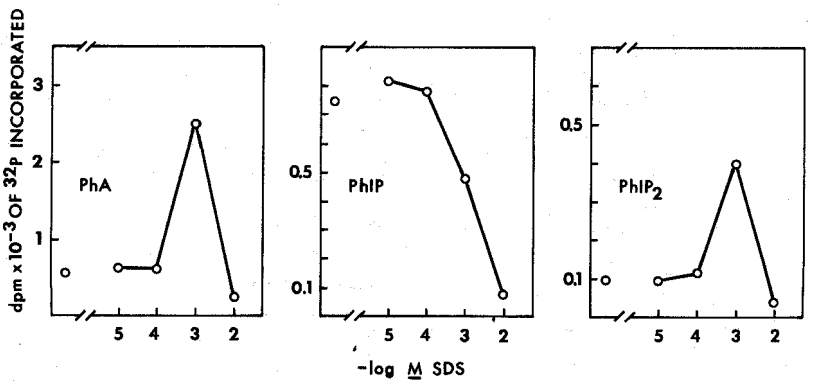

FIG. 3. The particulate fraction of guinea pig cerebral cortex (1 mg protein) was incubated for 5 min with 3 μCi [^{32}P]ATP as described in 'Methods'.

had been made (HOKIN & HOKIN, 1959; LAPETINA & HAWTHORNE, 1971). We found indeed that the shapes of the dose–response curves for TPB, DOC and SDS were similar, indicating a related action of TPB and the detergents on the labelling. Furthermore, the additional presence of TPB in incubations with DOC which is known to stimulate diglyceride kinase, did not produce an additional increase in labelling, again indicating that TPB action was not different from that of DOC.

TPB, although generally not looked upon as a detergent, has an anionic charge and hydrophobic phenyl groups, structural characteristics of amphiphatic molecules. The cell-dispersing properties of TPB which surpass those of other potassium-chelating agents (RAPPAPORT & HOWZE, 1966*b*) may indeed be aided by such weak detergent action in addition to its potassium-chelating properties. This idea is strengthened by the finding that TPB-dispersed cells are unable to carry on normal ribosomal protein synthesis (FRIEDMAN & EPSTEIN, 1967), and that TPB changes the permeability of the chloroplast membrane (HORTON & PACKER, 1968). Other properties of TPB which cannot be explained by its potassium-chelating capacity such as the inhibition of various enzymes (STANBURY & WICKEN, 1969) or the uncoupling of oxidative phosphorylation (UTSUMI & PACKER, 1967) should be reconsidered from the standpoint of detergent action. In any case, the stimulation of phospholipid labelling in brain by TPB appears to be due primarily to a detergent-like activation of diglyceride kinase and not due to the potassium-chelating or parasympathomimetic properties of this drug.

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REFERENCES

DURELL J., GARLAND J. T. & FRIEDEL R. O. (1969) *Science* **165,** 862–866.

DURELL J., SODD M. A. & FRIEDEL R. O. (1968) *Life Sci.* (II) **7,** 363–368.

FRIEDEL R. O. & SCHANBERG S. M. (1972) *J. Pharmacol. exp. Ther.* **183,** 326–332.

FRIEDMAN T. & EPSTEIN C. J. (1967) *Biochim. biophys. Acta* **138,** 622–624.

GLYNN I. M. & CHAPPEL J. B. (1964) *Biochem. J.* **90,** 147–154.

HAJRA A. K., SEGUIN E. B. & AGRANOFF B. W. (1968) *J. biol. Chem.* **243,** 1609–1616.

HARRIS C. C. & LEONE C. A. (1966) *J. Cell Biol.* **28,** 405–408.

HERZ F., KAPLAN E. & LUNA I. G. (1971) *Experientia* **27,** 1260–1261.

HOKIN L. E. (1969) In *Structure and Function of Nervous Tissue* (BOURNE G. H., ed.) Vol. 3, pp. 161–184. Academic Press, New York.

HOKIN L. E. & HOKIN M. R. (1958) *J. biol. Chem.* **233,** 822–826.

HOKIN M. R. & HOKIN L. E. (1959) *J. biol. Chem.* **234,** 1381–1386.

HOKIN M. R., HOKIN L. E. & SHELP W. D. (1960) *J. gen. Physiol.* **44,** 217–226.

HOLLANDER J., HALLENBECK J. M. & AGRANOFF B. W. (1969) *Biochem. biophys. Res. Comm.* **35,** 346–353.

HOLLANDER J., HALLENBECK J. M. & AGRANOFF B. W. (1970) *J. Neurochem.* **17,** 1247–1261.

HORTON A. A. & PACKER L. (1968) *Arch. Biochem. Biophys.* **128,** 820–823.

LAPETINA E. G. & HAWTHORNE J. N. (1971) *Biochem. J.* **122,** 171–179.

LOWRY O. H., ROSEBROUGH N. J., FARR A. L. & RANDALL R. J. (1951) *J. biol. Chem.* **193,** 265–275.

RAPPAPORT C. & HOWZE G. B. (1966*a*) *Proc. Soc. exp. Biol. Med.* **121,** 1010–1016.

RAPPAPORT C. & HOWZE G. B. (1966*b*) *Proc. Soc. exp. Biol. Med.* **121,** 1016–1021.

SCHACHT J. & AGRANOFF B. W. (1972*a*) *J. biol. Chem.* **247,** 771–777.

SCHACHT J. & AGRANOFF B. W. (1972*b*) *J. Neurochem.* **19,** 1417–1421.

SCHACHT J. & AGRANOFF B. W. (1973) *Biochem. biophys. Res. Comm.* **50,** 934–941.

SCHACHT J. & AGRANOFF B. W. (1974) In *Neurochemistry of Cholinergic Receptors* (DE ROBERTIS E. & SCHACHT J. eds.). Raven Press, New York.

SCHACHT J., NEALE E. A. & AGRANOFF B. W. (1974) *J. Neurochem.* in press.

SEIFTER J., GUIDERI G. & SEIFTER E. (1968) *Eur. J. Pharmacol.* **3,** 263.

STANBURY J. B. & WICKEN J. V. (1969) *Biochim. biophys. Acta* **178,** 403–405.

UTSUMI K. & PACKER L. (1967) *Arch. Biochem. Biophys.* **122,** 509–515.

WITTIG G., KEICHER S., RÜCKERT A. & RAFF P. (1949) *Annalen* **563,** 110–126.

YAGIHARA Y. & HAWTHORNE J. N. (1972) *J. Neurochem.* **19,** 355–367.

ZAHLER W. L., BARDEN R. E. & CLELAND W. W. (1968) *Biochim. biophys. Acta* **164,** 1–11.