Biochemical Pharmacology, Vol. 24, pp. 1013–1017. Pergamon Press, 1975. Printed in Great Britain.

INHIBITION OF CHOLINE ACETYLTRANSFERASE BY QUATERNARY AMMONIUM ANALOGUES OF CHOLINE

S. P. MANN and CATHERINE HEBB

Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, England

(*Received* 23 *September* 1974; *accepted* 3 *December* 1974)

Abstract—The effects of the choline analogues hemicholinium-3, triethylcholine, tetraethylammonium and acetylcholine on the synthesis of ACh by choline acetyltransferase have been studied *in vitro*. Hemicholinium-3 is shown to be an inhibitor of choline acetyltransferase with a K_i of 2·5 mM. HC-3, TEA and ACh are competitive with choline. Michaelis constants for HC-3 and TEC are also calculated and show that these two compounds are competitive substrates. A comparison of the *in vitro* with the known *in vivo* behaviour of these compounds is made and the probability stressed that their *in vivo* inhibitory effect on ACh synthesis is due to inhibition of ChAc itself. The results favour this explanation rather than the hypothesis that inhibition occurs because of interference with choline transport.

After showing that hemicholinium-3 (HC-3*) could be acetylated by choline acetyltransferase (acetyl-CoA: choline *O*-acetyltransferase EC 2.3.1.6, ChAc), Arnaiz *et al.* [1] suggested that this acetylation could be the cause of the inhibition of acetylcholine (ACh) synthesis which is readily demonstrated *in vivo*. However, it has been widely assumed that the inhibition is due to the inhibition of the choline uptake mechanism [2, 3] which would then in turn reduce the amount of choline available for ACh synthesis. Matthews [4] suggested that HC-3, triethylcholine (TEC) and tetraethylammonium (TEA) which are pharmacologically indistinguishable, may have the same action presynaptically. MacIntosh [5], however, quoting from Matthew's data, has argued that the reversal of the inhibition of these three compounds by choline occurs too slowly to be accounted for by a mechanism on the outside of the presynaptic membrane and that it is much more likely to be produced within the nerve ending.

An investigation has now been made of the effects of the three compounds, together with ACh, on the synthesis of ACh *in vitro* by a particle free preparation of ChAc from rat brains.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]acetyl-CoA was prepared using a method similar to that described by Morris *et al.* [6, 7]. CoA was obtained from P.L. Biochemicals, Milwaukee, U.S.A., and [1-^{14}C]acetic anhydride from the Radiochemical Centre, Amersham. HC-3 dibromide was obtained from Ralph N. Emanuel Ltd., Wembley, Middlesex, choline from B.D.H. Laboratory Chemicals Division, Poole, U.K., TEA bromide from Koch-Light, Colnbrook, Bucks. TEC chloride was a gift from Ward, Blenkinsop & Co., Liverpool and acetylcholine iodide (AChI) was a gift from Dr. D. Morris, University of Southampton. All other reagents were of the best available quality.

Choline acetyltransferase. This was prepared from acetone dried powders of rat brain, which were extracted (25 mg/ml) with 0·1 M phosphate buffer pH 6·5 containing 1 mM EDTA and 1 mM dithiothreitol. After freezing the extract for 24 hr at −18° it was spun at 90,000 *g* for 1 hr; 80 per cent of the ChAc was recovered in the supernatant. This was then run on a Sephadex® G25 column, which had been equilibrated with the extracting medium, to remove endogenous choline. The preparation was then concentrated in an Amicon Diaflo® chamber with a PM 30 membrane to a concentration equivalent to 50 mg/ml of the original acetone powder. This enzyme preparation when incubated in the absence of added choline synthesised less than 0·05 per cent of the ACh synthesised in the presence of added choline. Storage of the enzyme at −18° did not increase endogenous choline probably because any L-3-glycerylphosphorylcholine glycerophosphohydrolase (EC 3.1.4.2) present in the extracts was inhibited by the EDTA they contained.

Assay of enzyme. Choline acetyltransferase was assayed using the method described by Hebb *et al.* [7]. The basic incubation mixture consisted of 33 mM phosphate buffer pH 7·5, 0·5 mM EDTA, 1 mg/ml bovine plasma albumin and 0·13 M eserine sulphate. To this was added an amount of enzyme extract equivalent to 0·5 mg of the acetone powder prepared as described above. This incubate was found by flame photometry to contain 80 mM Na^+. Other constituents, acetyl-CoA (sp. act. 7–8 μCi/μmole), choline and choline analogues, as well as NaCl, were added as required and in the concentrations indicated in the results. With some exceptions the incubation was stopped after 15 min by the addition of 80 μl 0·15 M $HClO_4$ and cooling. The labelled products were separated from acetyl-CoA on a 6·5 × 100 mM column of Zerolit FF-IP resin, 100–200 mesh. What was taken to be acetylated HC-3 or acetylated TEC behaved in the same manner as acetylated choline on these columns. The radioactivity in the eluates from the

* *Abbreviations used.* ACh—acetylcholine; ChAc—choline acetyltransferase; CoA—coenzyme A; HC-3—hemicholinium-3; TEA—tetraethylammonium; TEC—triethylcholine.

columns was counted in Unisolve (Koch Light) in a Packard Liquid Scintillation Spectrometer.

Separation of ACh from acetyl-HC-3. After incubation the reaction was stopped by rapid cooling, and the incubate immediately placed on a 6·5 × 100 mm column of Amberlite CG50 resin (100–200 mesh in the sodium form). This resin does not function readily at low pH, and therefore acidification of the incubate was omitted. The column was washed with 5 ml H_2O to remove the acetyl-CoA while the products, acetyl-HC-3 and ACh remained bound. The ACh was then eluted with 10 ml 50 mM $MgCl_2$ which did not displace the acetyl-HC-3. The blanks for this method were higher than those obtained when Zerolit resin was used. This was probably due to some leakage of acetyl-HC-3 from the CG-50 resin.

Determination of K_m *and* K_i. Values for the enzyme inhibitor dissociation constants (K_i) were determined by assaying the ACh synthesised in the presence of 0·18 mM acetyl-CoA at concentrations of choline of 0·025, 0·05, 0·1 and 0·2 mM together with the choline analogues. The results were plotted as double reciprocals (i.e. with $1/v$ against $1/S$); to determine the nature of the inhibition; values for K_i were obtained from Dixon [8] plots ($1/v$ against I) which are applicable to competitive, non-competitive, and mixed inhibitors. Values for the Michaelis constant (K_m) were determined by incubating the same four concentrations of choline, or suitable concentrations of the analogue, with 0·18, 0·09, 0·045 and 0·0225 mM acetyl-CoA. Values for the apparent K_m were determined from double reciprocal plots, and values for K_m at saturating substrate concentrations from secondary plots of V_{max} against the concentration of the second substrate [6]. The results obtained were verified by statistical analysis according to Wilkinson [9]. In all cases initial rates obtained for the incubation time used (10 or 15 min).

RESULTS

The apparent inhibition of ACh synthesis by HC-3. The inhibition of ChAc by HC-3 was first tested with an enzyme preparation from which the endogenous choline had not been removed. Furthermore, the sodium concentration of the incubation medium was brought to 380 mM by the addition of NaCl (optimum for saturating substrates). Because of the endogenous choline in the enzyme, the inhibition of ACh synthesis (Fig. 1) was incomplete no matter how high the concentration of HC-3, and plotting double reciprocal plots did not produce straight lines. However, when from these values, values for ACh synthesis due to endogenous choline were subtracted, plots which indicated that nearly complete inhibition by HC-3 were obtained and the reciprocal plots were straight lines. These plots (Fig. 2) indicated that HC-3 was competitive with respect to choline and non-competitive with respect to acetyl-CoA.

To obtain inhibition of the order shown in Fig. 2, high concentrations of HC-3 had to be used but it was subsequently found that these concentrations were necessary only because the Na^+ level was also high.

The effects of sodium. The effects of sodium on the synthesis of ACh were examined using the enzyme, previously described, which had a very low concentration of choline. At high concentrations of NaCl and low concentrations of choline (0·025–0·20 mM) a marked inhibition by NaCl was observed. This occurred even though at saturating concentrations

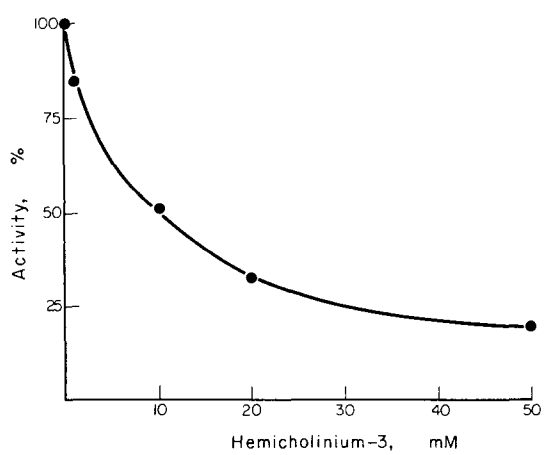

Fig. 1. Inhibition of ChAc by HC-3, in a preparation containing endogenous choline. Sodium concentration approx. 380 mM.

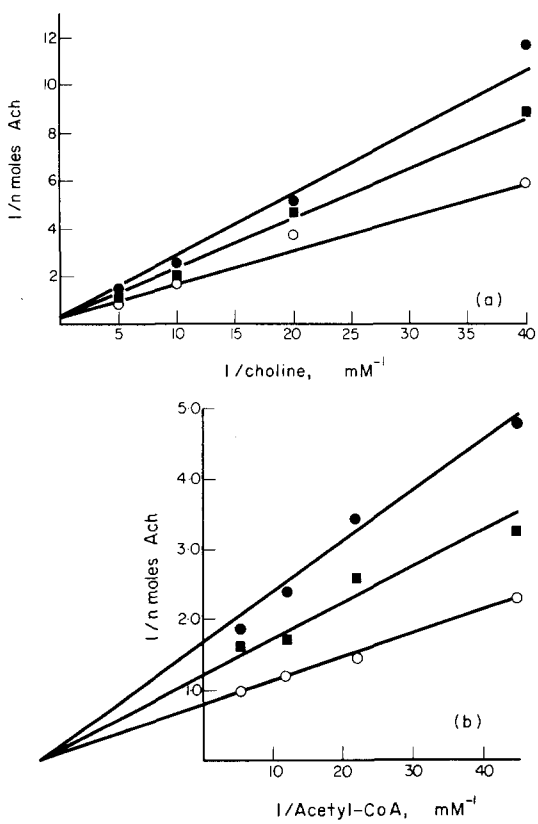

Fig. 2. In this and all subsequent figures the values plotted have been corrected by subtracting the values obtained when no choline or HC-3 had been added to the incubation mixture. (a) Double-reciprocal plot of HC-3 inhibition. Sodium concentration approx. 380 mM. ● 5 mM HC-3; ■ 2·5 mM HC-3; ○ No HC-3. HC-3 is competitive with choline. (b) Double-reciprocal plot of HC-3 inhibition. Sodium concentration approx. 380 mM. ● 20 mM HC-3; ■ 10 mM HC-3; ○ No HC-3. HC-3 appears to be non-competitive with acetyl-CoA.

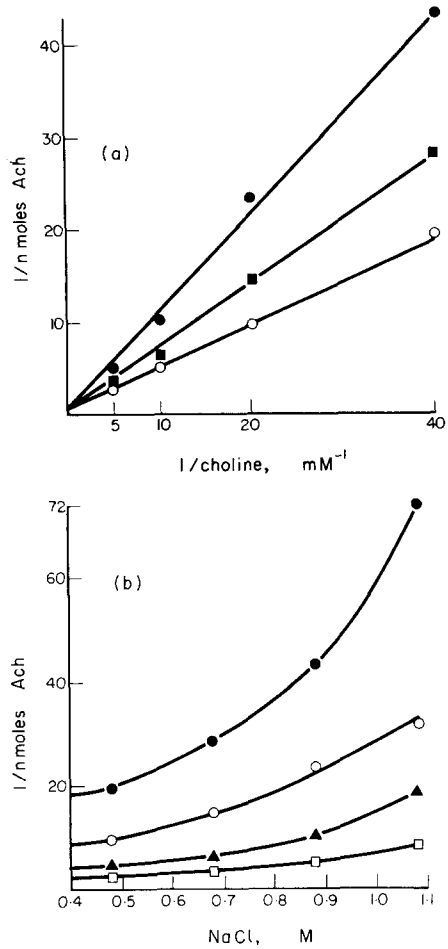

Fig. 3. (a) Double-reciprocal plot of Na^+ inhibition. ● 880 mM; ■ 680 mM; ○ 480 mM. Na ions appear to be competitive with choline. (b) Dixon plot of Na^+ inhibition. ● 0·025 mM choline; ○ 0·05 mM choline; ▲ 0·1 mM choline; □ 0·2 mM choline.

of choline, NaCl up to 400 mM was observed to activate the enzyme; at higher concentrations of NaCl some inhibition became apparent. Double reciprocal plots showed this inhibition to be competitive (Fig. 3a); but Dixon [8] plots gave the curved lines shown in Fig. 3b. This is normally regarded as evidence that more than one molecule of inhibitor is combining with the enzyme.

On examining the inhibitory effect of NaCl in respect to acetyl-CoA, the data obtained yielded parallel double reciprocal plots (normally indicative of uncompetitive inhibition). It is not obvious whether this was a genuine case of uncompetitive inhibition, or whether it was due to the occurrence of some activation, which may have been related to the interaction of acetyl-CoA with the enzyme, mixed with the predominant inhibition. Only slight inhibition was observed when the concentration of Na^+ was 130 mM (80 mM + 50 mM NaCl) and that of choline 0·1 mM. Subsequent determinations were therefore done without addition of NaCl (i.e. at 80 mM Na^+).

Determinations of K_i. K_i values were determined for AChI, TEA bromide, and for HC-3 at 80 mM Na^+ and 0·18 mM acetyl-CoA (0·5 mM is saturating). The values obtained from Dixon plots are given in

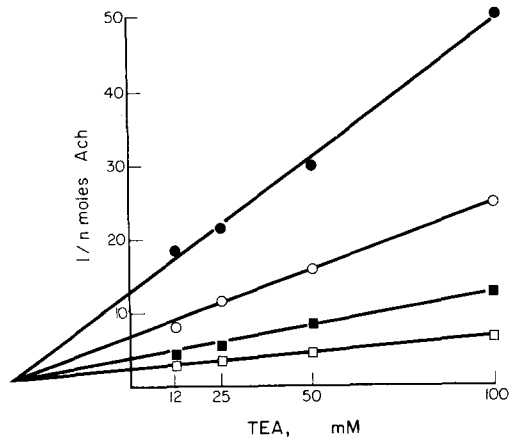

Fig. 4. Dixon plot of TEA inhibition. ● 0·025 mM choline; ○ 0·05 mM choline; ■ 0·1 mM choline; □ 0·2 mM choline. The point of intersection of the lines shows the K_i to be 32·4. Na^+ concentration was 80 mM.

Table 1; the plot for TEA is shown in Fig. 4. All four compounds were found to be inhibitory and competitive with choline. The K_i for HC-3 was considerably lower at 80 mM Na than the value obtained at higher levels of sodium. The inhibitory action of HC-3 was also much more powerful and it became necessary to lower its concentration considerably below the levels used previously (as in Fig. 2); otherwise it was too inhibitory to produce satisfactory plots.

The determination of K_m *values.* The K_m values presented a problem, particularly for choline, at

Table 1. Choline acetyltransferase; values for K_m and K_i of choline and its analogues

Substance	Constant	Value (mM)
Choline chloride	K_m	0·29 ± 0·02
HC-3 bromide	K_m	2·9
	K_i	2·5
ACh iodide	K_i	22·5
TEA bromide	K_i	32·4
TEC chloride	K_m	50·0

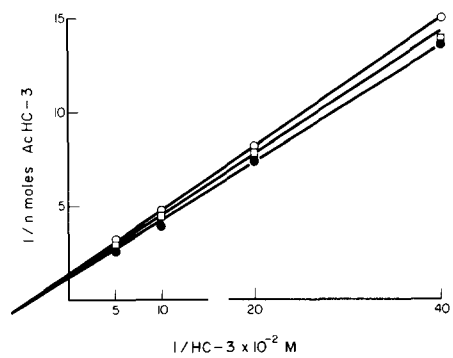

Fig. 5. Double-reciprocal plot of HC-3 acetylation, ○ 0·18 mM acetyl-CoA; □ 0·09 mM acetyl-CoA; ● 0·045 mM AcCoA. The apparent K_m's were not significantly different from the K_m at saturating acetyl-CoA calculated from a secondary plot of these data. Na^+ concentration was 80 mM.

80 mM Na^+ for unlike the plots shown by other authors [6] in higher Na levels the lines produced by double-reciprocal plots appeared very close together (Fig. 5 shows the plot for HC-3). Values for the apparent K_m of choline from such a plot were not significantly different from each other, nor were they significantly different from the value obtained by means of a secondary plot of the value of $1/v$ at saturating concentration of acetyl-CoA. This was also true for HC-3 and TEC. This made the determination of a K_m for acetyl-CoA at saturating choline values very difficult, but from these plots it can be concluded that it was not greater than 10 μM.

The difference in the results described here from those obtained by Morris *et al.* [6] can be ascribed to the difference in sodium concentration. The apparent K_m for choline in Fig. 2, at 380 mM Na^+, was found to be 0·6 mM whereas at 80 mM NaCl, the lower level of Na^+, it fell to 0·2 mM. This value is considerably lower than K_m values obtained by a number of authors who have carried out their measurements at higher salt concentrations.

DISCUSSION

Although it was originally thought that the action of HC-3 *in vivo* might be due to a direct effect on choline acetyltransferase, the apparently weak effect of this compound on ChAc in solution led MacIntosh and coworkers to suggest that it might act by inhibiting choline transport [10, 11]. Using squid giant axons Hodgkin and Martin [12] have shown that the choline uptake by this tissue is inhibited by HC-3, ACh, TEC, and TEA with inhibitory constants of 0·02, 0·1, 2 and 10 mM respectively. Gardiner [13] added further to the transport hypothesis when he showed that the ACh synthesis by untreated homogenates of brain was inhibited by HC-3 while ether treated homogenates were unaffected.

Another possible explanation of the inhibitory action of HC-3 is based on the observation that HC-3 itself can be acetylated [1]; the experiments described in this paper are consistent with that idea. When low choline and high HC-3 concentrations were incubated together the total radioactivity appearing in the eluted samples was higher than in the control samples without HC-3. The labelled acetyl groups of acetyl-CoA are the only possible source of this activity which was also dependent on the presence of active enzyme. The additional activity was absent when HC-3 was omitted, and most clearly apparent when choline but not HC-3 was omitted. The HC-3 molecule appears able therefore to accept acetyl groups in a reaction catalyzed by ChAc; it seems most likely that the reaction which occurs is acetylation of either or both of the hydroxyl groups of HC-3 in competition with choline.

However, it is clear that the acetylation of HC-3 is considerably modified by the Na^+ concentration at which it is incubated with the enzyme. Our tests show that the acetylation at 380, as compared with 80 mM Na^+, is inhibited to a much greater degree. This could be part of the reason why its direct action on ChAc has not been convincingly demonstrated in the past, since those who have studied the extracted enzyme have generally been concerned to establish optimal conditions for its activity which means working at high K^+ or Na^+ levels. It is probable, however, that the normal combined intracellular levels of Na^+ and K^+ are nearer to 80 mM than to 380 mM. If so, it would help to account for the differences between the responses to HC-3 *in vivo* and *in vitro* that have generally been observed.

In this connection it is of interest to consider further the question of how far the effectiveness of HC-3 and other choline analogues in inhibiting ChAc *in vivo* can be related to the inhibition seen *in vitro*. It is not possible to compare the K_m and K_i directly without first considering the meaning of the K_i obtained. Of the four compounds examined only two (ACh and TEA) are classical competitive inhibitors, the other two (HC-3 and TEC) must rather be regarded as competitive substrates. The K_i obtained for HC-3 therefore is not a true K_i because it contains an additional constant for the dissociation of the enzyme–substrate complex into products (acetyl-HC-3 and CoA). The observed K_m for HC-3, however, is indistinguishable from the K_i and it therefore seems reasonable to compare these values with the values for K_m and K_i of the other analogues. The K_m for TEC can also be compared with the K_i value because in this case the K_m is over 200 times the K_m for choline and the constant for the dissociation of enzyme–TEC complex is consequently very small, and can reasonably be ignored for the purpose of comparing the effectiveness of the inhibitors. These considerations make it possible to arrange the inhibitors in the order of their effectiveness (see Table 1). From this it can be seen that HC-3 is the most potent and it would appear that the –OH groups of the substrate molecule play an important role in binding inhibitor or substrate to the enzyme; while the replacement of the methyl groups on the quaternary nitrogen with ethyl groups, reduces affinity at this end of the molecule.

In experiments using HC-3, TEA and TEC on the perfused superior cervical ganglion, Matthews [4] was able to show that these compounds all reduce ACh synthesis in the intact ganglion, but the amount of TEC and TEA required to produce 90 per cent inhibition was 10 times greater than the required amount of HC-3. Choline added to the perfusate reduced the inhibition and perfusion with inhibitor-free plasma eventually reversed the inhibition completely, although this took up to 1 hr. MacIntosh [5] has pointed out that ACh analogues which affect the postsynaptic region, are rapidly removed by a change in perfusion medium. He therefore suggests that the mechanism of inhibition is unlikely to be uptake of choline in the presynaptic membrane, but rather an intraterminal mechanism, such as competition with ACh for the ACh binding sites. Bowman and Rand [14] have suggested that acetylated TEC may be released as a false transmitter, but while this may happen it is not necessary for acetyl-TEC to be released for failure in transmission to occur.

The carrier-mediated uptake of choline is inhibited by HC-3 which has a K_i of about 4 μM [2]. TEA and TEC were shown to be 125 times less potent than HC-3. The theory that this inhibition is responsible for the inhibition of ACh synthesis, is based on the assumption that a reduction in the intraterminal choline uptake automatically reduces the ACh synthesis. The work of Ansell and Spanner [15] how-

ever, indicates that the choline taken up is preferentially converted to choline phosphate with little from this source being available for ACh synthesis. Inhibition of the uptake process would therefore be expected to have relatively less effect on ACh synthesis than would direct inhibition of the synthesising enzyme.

The evidence presented here has shown that a number of quaternary ammonium compounds can inhibit the synthesis of ACh by a direct action on the enzyme choline acetyltransferase. The K_i values for TEA and TEC are, however, 10–20 times greater than the K_i for HC-3 which is very close to the 10-fold difference that Matthews observed with TEA, TEC as compared with HC-3 in his experiments. Both results are numerically incompatible with the results obtained by Hodgkin and Martin [12] in the investigation already quoted on the specific uptake process of the axon. This increases the probability that they inhibit ACh synthesis primarily by direct action on choline acetyltransferase. The effects *in vivo* of the analogues will depend to a considerable extent on the intracellular concentration of choline itself as would be expected from their competitive nature. If the concentration of choline is high, then little ChAc inhibition will occur; conversely if the concentration is low more inhibition will occur. The absolute values obtained for K_i and K_m are therefore valuable only as a comparison between themselves. Recent work, however, does indicate that free choline values for the rat brain are lower than originally reported [16–18]. This makes it all the more probable that inhibition *in vivo* of ACh synthesis by HC-3 does depend on its action as a competitive substrate of the synthesizing enzyme. This would still be true even if some of the HC-3 binds to subcellular organelles.

Acknowledgements—We are grateful to Mrs. Janet Hood and Mrs. Sally Rochead for their expert assistance in preparing the MS of this paper and to Mr. Kevin Lindsay for his help in some of the experimental work.

REFERENCES

1. G. Rodrigues de Lores Arnaiz, L. M. Zieher and E. De Robertis, *J. Neurochem.* **17,** 221 (1970).
2. I. Diamond and E. P. Kennedy, *J. biol. Chem.* **244,** 3528 (1969).
3. F. Fonnum, *Brain Res.* **62,** 497 (1973).
4. E. K. Matthews, *Br. J. Pharmac.* **26,** 552 (1966).
5. F. C. MacIntosh, *Can. J. Biochem. Physiol.* **41,** 2555 (1963).
6. D. Morris, A. Maneckjee and C. Hebb, *Biochem. J.* **125,** 857 (1971).
7. C. O. Hebb, S. P. Mann and J. Mead, *Biochem. Pharmac.* **24,** 1007 (1975).
8. M. Dixon and E. C. Webb, *Enzymes*, 2nd Edn. Longmans, London (1964).
9. G. N. Wilkinson, *Biochem. J.* **80,** 324 (1961).
10. F. C. MacIntosh, R. R. I. Birks and P. B. Sastry, *Nature, Lond.* **178,** 1181 (1956).
11. F. C. MacIntosh, *Can. J. Biochem. Physiol.* **37,** 343 (1959).
12. A. L. Hodgkin and K. Martin, *J. Physiol. (Lond).* **179,** 26P (1965).
13. J. E. Gardiner, *Biochem. J.* **81,** 297 (1961).
14. W. E. Bowman and M. J. Rand, *Br. J. Pharmac.* **17,** 176 (1961).
15. G. B. Ansell and S. Spanner, *Biochem. J.* **110,** 201 (1968).
16. K. Dross and H. Kewitz, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **274,** 91 (1972).
17. I. Eade, C. Hebb and S. P. Mann, *J. Neurochem.* **20,** 1499 (1973).
18. P. A. Shea and M. H. Aprison. *Analyt. Biochem.* **56,** 165 (1973).