Biochemical Pharmacology, 1961, Vol. 6, pp. 195–204. Pergamon Press Ltd., Printed in Great Britain.

EFFECTS OF ACETYLCHOLINE ANALOGUES AND ETHANOL ON THE RESPIRATION OF BRAIN CORTEX TISSUE *IN VITRO*

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(*Received* 8 *September* 1960)

Abstract—Unstimulated and electrically stimulated brain cortex slices were exposed to compounds related to acetylcholine, and to Triton, with or without $1{\cdot}96 \times 10^{-1}$ M (0·9 per cent) ethanol.

The lipid-insoluble substances acetylcholine, curare, deca- and suxa-methonium were inactive on the brain tissue respiration. In unstimulated tissue, the lipid-soluble acetylcholine analogues pyridine-2-aldoximedodecyl iodide (PAD) and cetyltrimethylammonium bromide (Cetavlon), like ethanol, caused a transient increase in respiration. Cetavlon, cetylpyridine chloride (CPC) and Triton reduced the respiration of both stir ulated and unstimulated tissue at approximately identical concentrations, the relative effect on unstimulated tissue being smaller. 10^{-4} M PAD and $1{\cdot}8 \times 10^{-3}$ M atropine abolished the response to stimulation almost completely leaving unstimulated tissue unaffected. Ethanol significantly increased the effect of atropine, PAD, Cetavlon and CPC on stimulated tissue. The non-ionized detergent Triton was not synergistic with ethanol.

In intact rats, PAD potentiated ethanol action on the CNS Cetavlon acted similarly, but caused strong haemolysis when injected with ethanol. *In vitro*, haemolysis caused by Cetavlon was not increased by low ethanol concentrations.

The results are consistent with the hypothesis that the acetylcholine system is involved in the reaction of brain slices to electrical stimulation, and indicate that ethanol may depress the functional response to stimulation by unspecific action on this system.

ETHANOL at concentrations associated with intoxication *in vivo* increases the respiration of unstimulated brain slices at least transiently[1, 2] and depresses that of brain tissue stimulated with potassium ions[2, 3] or with applied electrical pulses.[3, 4] As stated by Hunter and Lowry,[5] in seeking an explanation for the mechanism of anaesthesia, the crucial problem is perhaps to decide between two alternative possibilities: inhibition of a sensitive energy-yielding reaction essential for the functioning of nerves, or interference with specific transmission mechanisms. Work in these laboratories[3, 4] and by Beer and Quastel[6, 7] with brain cortex slices, and electrophysiological and metabolic evidence obtained with peripheral nerves,[8–11] has led the author tentatively to adopt the view that the depression of respiration in stimulated brain tissue exposed to ethanol is a secondary effect due to inhibition of the functional response. Wallgren and Kulonen[3] have also suggested that the effect of ethanol in increasing the respiration of unstimulated tissue may be due to its well established depolarizing action.

Rowsell[11a] has demonstrated that combined acetylcholine is lost from cerebral cortex slices during electrical stimulation. During recent years, Nachmansohn's theory of the essential role of the acetylcholine system in the excitation cycle of

conducting membranes (cf. the recent monograph[12]) has gained support from experiments with analogues of acetylcholine made lipid-soluble by substitution of one of the methyl groups with a long aliphatic chain. Some of the physiological effects of such a compound (PAD) were first described by Wilson[13], and most of the results obtained thus far have been reviewed.[12] Accepting Nachmansohn's theory and supposing that the neurones are activated in brain slices during electrical stimulation, one would expect lipid-soluble quaternary ammonium ions selectively to depress the respiratory response to stimulation. Furthermore, such compounds would be expected to cause at least a transient increase in the respiration of unstimulated slices. In the present investigation, these problems were studied, and also the relationship between the effect of ethanol and any changes possibly induced by the quaternary ammonium compounds.

Some of the compounds used were strong detergents, and their effects on brain tissue were compared with those of a non-ionized detergent. Haemolysis was used as a simple test of surface activity in a biological system and the results contrasted with those obtained with brain tissue in order to distinguish unspecific surface action from effects on systems specifically related to the function of nerve tissue.

MATERIALS AND METHODS

Compounds tested

Acetylcholine chloride (Hoffmann–La Roche); decamethonium iodide (L. Light & Co.); suxamethonium iodide grade C (Cal. Corp. Biochem. Res.); D-tubocurarine (Theodor Schuchardt, G.m.b.H.); pyridine-2-aldoximedodecyl iodide (PAD, obtained through the courtesy of a local laboratory); pyridine-2-aldoximemethyl iodide (PAM, Dajac); cetylpyridine chloride (CPC, AS Bionova); cetyltrimethylammonium bromide (Cetavlon, Fluka AG.); the alkylarylpolyethoxy alcohol Triton X-100 (Rohm & Haas Co.); racemic atropine sulphate (Bidle Sawyer & Co.); eserine sulphate (C. F. Boehringer & Soehne G.m.b.H.).

Experiments with brain cortex slices

Apparatus. All experiments were performed with conventional Warburg constant-volume respiratory manometers. A detailed description of the stimulators and electrode flasks has been given previously.[3] The only change in the equipment was that the earlier vibrators (with the frequency 90–100 c/s) were exchanged for new ones (Philips 7934/02; with the frequency 50 c/s) giving a pulse frequency of 100/sec. However, neither the stability nor the magnitude of the response was altered. A voltage gradient of 1·5–1·6 V/mm as measured with an electronic voltmeter was maintained between the electrodes.

Experimental conditions. Phosphate buffered medium[14] with 6 mM glucose was used throughout, the volume per flask being 2·5 ml. The gas phase was pure oxygen and the experimental temperature 37·5 °C.

Adult rats of the laboratory stock were used. The preparation of the slices and the general procedure have been described.[3] All inhibitors were dissolved in the experimental medium, and the solutions were placed in the side-arm of the flasks after gassing.

In some of the experiments, respiration was measured during a 20-min initial control period, after which the inhibitors were tipped into the main vessel and the recording of respiration continued from 25 to 85 min after the first reading. Any change in

respiration relative to the first 20-min period was thus observed. In other experiments, the inhibitors were added during the 5-min period between initiation of stimulation and commencement of manometer reading. The manometers were read at 10-min intervals.

The experiments were usually set up with two standard flasks and two electrode flasks, although preliminary experiments were often made with unstimulated tissue only. When pulses were applied, one slice (20–40 mg) from each brain hemisphere was used for measuring respiration of unstimulated tissue, and two slices (30–50 mg) for that of stimulated tissue. The two pairs of flasks used in each experiment were thus provided with slices obtained from different lobes of the same brain and subjected to different conditions with respect to the inhibitor present.

All compounds were tested in conjunction with ethanol: these experiments were always performed with one pair of flasks with the inhibitor only and the other pair with the same concentration of the inhibitor in the presence of $1{\cdot}96 \times 10^{-1}$ M (0·9 per cent) ethanol. Our earlier experiments with ethanol[3, 4] were supplemented by measuring the effects of higher concentrations of ethanol.

Experiments with intact animals

The effect of PAD, PAM and Cetavlon on ethanol intoxication in rats was measured by means of the "tilted plane" test developed in these laboratories.[15] In testing, the animal is placed, facing up the slope, on an inclined plane with a rough surface, the plane is tilted and the angle at which the animal slides down is recorded. The results are expressed as percentages of an initial sober value obtained immediately before the administration of alcohol.

The animals were fasted overnight and the alcohol was injected intraperitoneally as a 10 per cent (w/v) solution in saline. PAD, PAM and Cetavlon, respectively, were dissolved in this solution when given together with ethanol. When given alone, PAM and Cetavlon were dissolved in saline, PAD in propylene glycol. Propylene glycol in the dose employed (2·5 ml/kg) did not affect the performance of the rats.

In each series of experiments, the animals were of the same age and sex.

Experiments on haemolysis

The haemolytic effect of Cetavlon and Triton on whole blood and on washed red cells was measured in the absence and presence of ethanol. The experiments were carried out at room temperature. The compounds tested were dissolved in the experimental medium used for the brain slices. This was also used for washing and suspending red cells. In experiments with whole blood, the amount of medium and detergent added was 4 per cent of the total volume. The compounds tested were allowed to act on the blood cells for 20 min, including the centrifugation time. The colour developed was measured with a spectrophotometer at 540 mμ.

RESULTS

Respiration of brain slices

In Table 1, the results of experiments with a 20-min control period and subsequent measurement of the drug effects are summarized. For the active compounds, the lowest concentration with a clear depressing effect on the respiration of stimulated tissue is given. The physiologically highly active but lipid-insoluble quaternary ammonium compounds acetylcholine, curare, deca- and suxa-methonium were inactive

in the conditions used. By contrast, Cetavlon and CPC, both containing a long aliphatic chain, depressed respiration at very low concentrations. In addition Cetavlon caused a transient increase in the respiration of unstimulated tissue, an effect which was not found with CPC.

TABLE 1. EFFECTS OF VARIOUS COMPOUNDS ON OXYGEN CONSUMPTION OF BRAIN SLICES IN THE EXPERIMENTS WITH AN INITIAL CONTROL PERIOD

The symbols indicate: (a) no effect; (b) depression; (c) response to stimulation completely abolished; (d) transient increase; (O) no modification of the effect in presence of ethanol; X, depression increased in presence of ethanol.

Compound	Concentration range tested (M)	Effect on oxygen consumption		Synergism with ethanol
		Stimulated tissue	Unstimulated tissue	
Acetylcholine	$1{\cdot}1 \times 10^{-2}$	(a)	(a)	(O)
Eserine	$3{\cdot}1 \times 10^{-5}$–$1{\cdot}5 \times 10^{-3}$	(a)	(a)	(O)
Acetylcholine + eserine	$1{\cdot}1 \times 10^{-2}$ + $3{\cdot}1 \times 10^{-5}$	(a)	(a)	(O)
Curare	$2{\cdot}9 \times 10^{-3}$	(a)	(a)	(O)
Decamethonium	$3{\cdot}9 \times 10^{-5}$–2×10^{-3}	(a)	(a)	(O)
Sux-amethonium	$3{\cdot}9 \times 10^{-5}$–2×10^{-3}	(a)	(a)	(O)
Cetavlon	$5{\cdot}5 \times 10^{-5}$–$1{\cdot}4 \times 10^{-3}$	(b), (c) at $5{\cdot}5 \times 10^{-3}$	(b), (d) at $5{\cdot}5 \times 10^{-3}$	X
CPC	$1{\cdot}5 \times 10^{-5}$–$4{\cdot}7 \times 10^{-4}$	(b), (c) at $4{\cdot}4 \times 10^{-4}$	(b)	X
Triton	15–1000 μg/ml	(b), (c) at 150 μg/ml	(b) from 40 μg/ml	(O)

Since Cetavlon and CPC are strong detergents and their effects might be due to unspecific surface activity, the non-ionized detergent Triton was used for comparison. In the present conditions, the effects were similar to those of CPC. Triton even proved to be rather more selective than Cetavlon or CPC in depressing the respiration of stimulated tissue.

Ethanol clearly augmented the depressing action of Cetavlon and CPC, but not that of Triton. However, the synergism between ethanol and Cetavlon or CPC, respectively, was not very strong, the effects being less than additive. The statistical significance of the increased depression in the presence of ethanol was $P < 0{\cdot}05$ for both compounds.

In 1-hr experiments in which the inhibitor was added immediately after the commencement of stimulation, the tissue respiration was considerably more sensitive to the inhibitor, and there was also a clearer tendency to selective action on the stimulated tissue. Results of 1-hr experiments are presented in Table 2. All the compounds listed had a highly significant depressing action on the respiration of stimulated tissue. Only one, Cetavlon, significantly depressed the respiration of unstimulated tissue ($P < 0{\cdot}01$). As indicated, ethanol increased respiration in the absence of stimulation. The series with ethanol is from an earlier study,[4] but is included here since the oxygen uptake of the previous control series (198 μmoles/g fresh tissue/hr) did not deviate significantly from the present controls. The experiments with Cetavlon and Triton gave results consistent with those obtained earlier, except that Cetavlon had a stronger effect on stimulated tissue (−37 per cent) than on unstimulated tissue (−14 per cent). There was an initial increase in the respiration of unstimulated tissue.

The synergism of ethanol with Cetavlon was highly significant: with Triton, there was again no synergism. Atropine, a tertiary compound, also depressed respiration, but was considerably less effective than Cetavlon. However, with $1{\cdot}8 \times 10^{-3}$ M, there was no response to stimulation, whereas unstimulated tissue did not react to the presence of atropine. Ethanol was clearly synergistic with atropine.

TABLE 2. OXYGEN CONSUMPTION OF RAT BRAIN CORTEX SLICES IN THE 1-HR EXPERIMENTS
The oxygen consumption is expressed as μmoles/g fresh tissue/hr ± standard deviation. $P <$ shows P-values for significance of the action of ethanol. Control and ethanol refer to different experimental series for which statistical evaluation was performed by means of Student's t-test. In all other series, the two hemispheres of the same brain were used in parallel experiments with identical concentrations of inhibitor, but the tissue from one of the hemispheres was exposed to ethanol, that from the other was not. The significance of the effect of ethanol was calculated by means of the t-test for correlated measures.

Experimental conditions	Stimulated tissue			Unstimulated tissue		
	No. of expts.	Mean oxygen consumption	$P <$	No. of expts.	Mean oxygen consumption	$P <$
Control	21	204 ± 17		31	125 ± 16	
Ethanol $1{\cdot}96 \times 10^{-1}$ M	21	167 ± 12	0·001	21	139 ± 15	0·005
Cetavlon $1{\cdot}1 \times 10^{-4}$ M	9	129 ± 16		9	107 ± 15	Not
Cetavlon + ethanol	8	105 ± 12	0·001	9	95 ± 19	sig.
Triton 15 μg/ml	9	141 ± 12	Not	9	119 ± 9	Not
Triton + ethanol	8	135 ± 6	sig.	9	114 ± 8	sig.
Atropine $6{\cdot}9 \times 10^{-4}$ M	6	162 ± 8		6	115 ± 12	Not
Atropine + ethanol	6	142 ± 20	0·005	6	122 ± 8	sig.
PAD $2{\cdot}4 \times 10^{-5}$ M	10	180 ± 8		11	125 ± 11	Not
PAD − ethanol	10	156 ± 12	0·005	11	117 ± 16	sig.
PAD $4{\cdot}8 \times 10^{-5}$ M	8	165 ± 6		10	128 ± 13	Not
PAD − ethanol	8	140 ± 16	0·025	10	125 ± 19	sig.

The most important results were those obtained with PAD, which even at a very low concentration markedly depressed the respiration of stimulated tissue, whereas that of unstimulated tissue was unaffected. PAD at $4{\cdot}8 \times 10^{-5}$ M was about as effective as ethanol at $1{\cdot}96 \times 10^{-1}$ M. PAD was also synergistic with ethanol in stimulated tissue, the effects being almost additive. The difference between the series with ethanol alone and with $2{\cdot}4 \times 10^{-5}$ M PAD + ethanol appeared rather small, but it was statistically significant ($P < 0{\cdot}05$). The finding with the higher concentration corroborated this point.

In Fig. 1, the effect of increasing concentrations of PAD on respiration is shown graphically. At $9{\cdot}6 \times 10^{-5}$ M PAD, the respiratory reaction to stimulation had almost disappeared whereas the respiration of unstimulated tissue was scarcely affected. However, the respiratory response to stimulation was never immediately abolished: it was noticeable during the first 20 min of stimulation even with the highest concentrations of PAD employed. In Fig. 2, the effect on respiration of varying concentrations of ethanol is presented. The graphs for PAD and ethanol are very similar: however, PAD is about 4000 times more effective than ethanol as an inhibitor of respiratory response to stimulation. As with PAD, there was an initial weak response to stimulation also with the highest concentration of ethanol used.

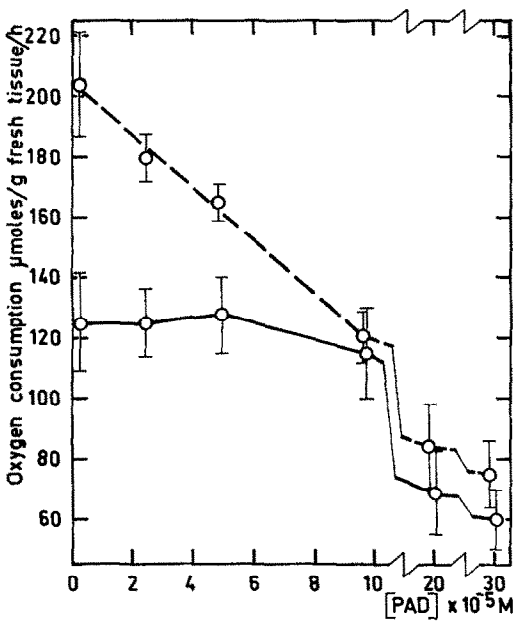

FIG. 1. Effect of PAD on respiration of brain cortex slices. Stimulated tissue (– – –), unstimulated tissue (———). The data in Table 2 concerning controls and PAD are included, the other points represent five determinations each. Vertical bars indicate standard deviation.

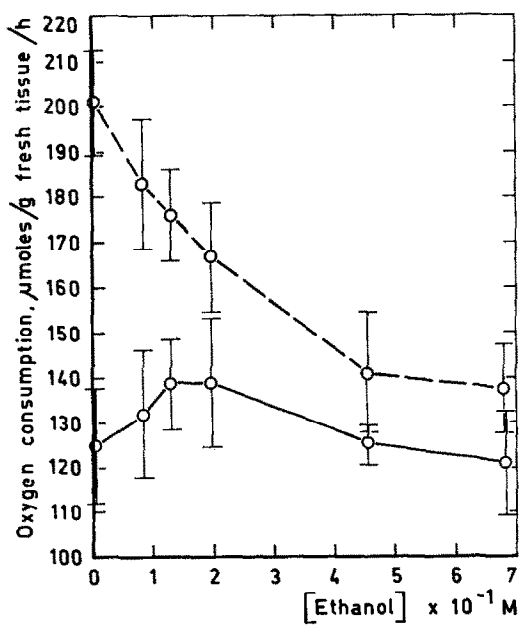

FIG. 2. Effect of ethanol on respiration of brain cortex slices. Stimulated tissue (– – –), unstimulated tissue (———). Each point represents the average of at least ten determinations. Vertical bars indicate standard deviations.

In Fig. 3, the time course of the oxygen consumption in various conditions is presented. When PAD was added to unstimulated tissue, the respiration rose initially well above and later fell slightly below that of the controls. This was a parallel to the observations with Cetavlon. With ethanol the respiration was elevated throughout the experimental period of 1 hr. However, when ethanol was added after 20 min,

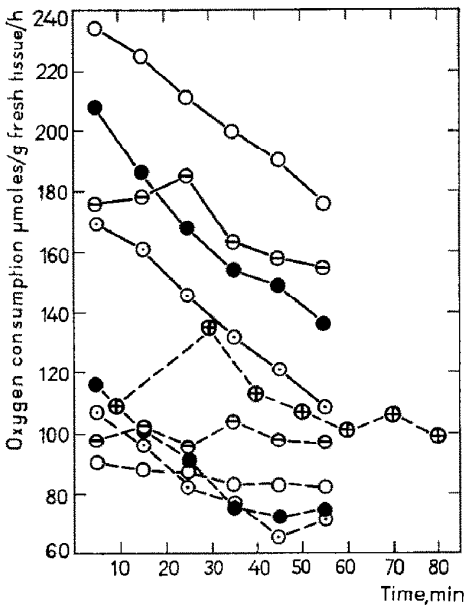

FIG. 3. Time course of oxygen consumption of brain cortex slices. Stimulated tissue (———), unstimulated tissue (– – –). Control medium (○); $4{\cdot}8 \times 10^{-5}$ M PAD (●); $1{\cdot}96 \times 10^{-1}$ M ethanol (⊖); PAD + ethanol (⊙); unstimulated tissue exposed to ethanol after initial 20-min control period (⊕) To avoid overlapping, the ordinates for 1-hr experiments with unstimulated tissue have been reduced by 40 μmoles and those for experiments with initial control period by 30 μmoles. Each curve represents the average of at least eight determinations.

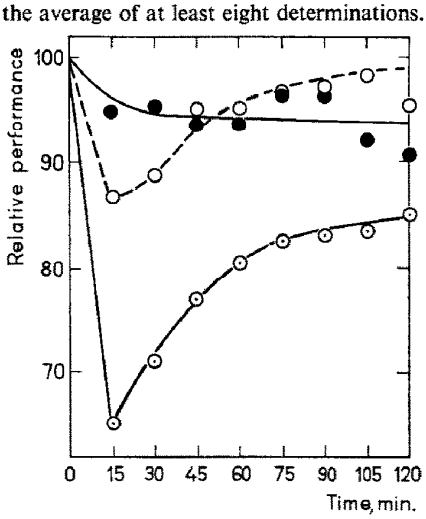

FIG. 4. Time course of intoxication in rats. Ethanol 1·5 g/kg (○); PAD 15 mg/kg (●); ethanol + PAD (○). Each group comprised ten individuals.

respiration increased transiently and then returned to the initial level. PAM in concentrations up to 5×10^{-3} M did not influence respiration.

Intoxication in intact animals

Of thirty female rats, ten received 1·5 g ethanol/kg, ten 15 mg PAD/kg, and ten 1·5 g ethanol + 15 mg PAD/kg in one injection. The time course of intoxication as measured during 2 hr following injection is shown in Fig. 4. The means of the lowest values observed indicated that ethanol reduced performance by 15·5 ± 4·4 per cent, PAD by 11·5 ± 3·7 per cent, and ethanol + PAD by 35 ± 5·8 per cent (results ± standard deviation). PAD thus potentiated the effect of ethanol. With PAD alone, the animals appeared curiously sleepy and depressed, but not inco-ordinated in the same way as with ethanol. PAM in the same molar dose had no observable effects when given alone and did not modify ethanol intoxication.

Cetavlon in a dose of 100 mg/kg approximately doubled the effect of 2 g/kg ethanol during the initial phase of intoxication. The animals treated (five males) became exanotic after about 90 min, and died of haemolysis within 2–2½ hr after injection. Five males injected with the same dose of Cetavlon but no ethanol were not overtly intoxicated and there were only slight signs of haemolysis. No other untoward effects were observed.

Haemolysis

The observation of haemolysis in intact animals treated with Cetavlon and ethanol prompted *in vitro* measurements of the haemolytic effect of Cetavlon. When ethanol

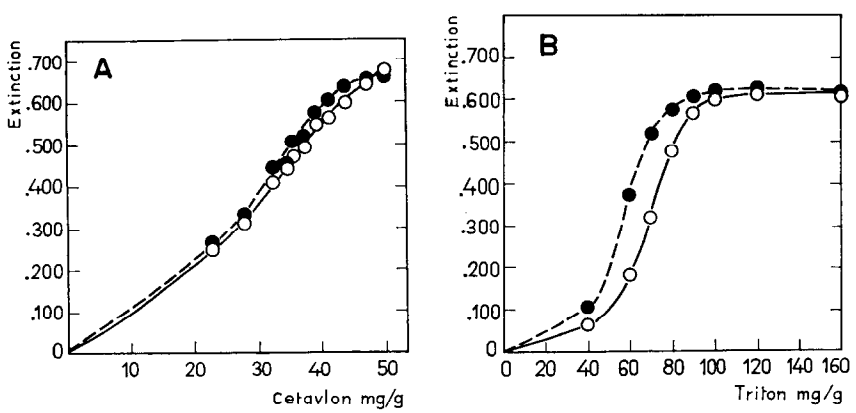

FIG. 5. Haemolysis of washed red cells in presence of (A) Cetavlon, (B) Triton. Cetavlon (Triton) only (○); Cetavlon (Triton) + 0·9 per cent ethanol (●). The curves for Cetavlon are averages of three series of measurements, those for Triton of two series.

was added at a concentration (0·3 per cent) approximately corresponding to the highest concentration to be expected in the blood of the intact animals, no effect on the haemolysis caused by Cetavlon was observable either in whole blood or in washed red cells. Thus ethanol did not directly augment the haemolysis in the intact animals but apparently facilitated the uptake of Cetavlon from the intraperitoneal cavity.

The haemolytic effect of Cetavlon and Triton was then tested *in vitro* with and without addition of 0·9 per cent ethanol in order to obtain effects which could be compared with those observed in brain tissue. The results (Fig. 5) indicate that in this preparation ethanol clearly augmented the effect of Triton, whereas that of Cetavlon was only slightly modified.

DISCUSSION

The lack of effect on respiration of acetylcholine and eserine in the present experiments corroborates the earlier findings of McIlwain[16]. The inactivity of curare, suxamethonium, decamethonium, and PAM is consistent with this observation. In accordance with Nachmansohn, one may assume that acetylcholine and related lipid-insoluble compounds fail to penetrate to the sites of action. These drugs seem to affect conduction at the points of transmission and are known to do so centrally also, for instance after topical application. Perhaps the explanation of the lack of effect on the stimulated slices is that the condensor discharges lead to simultaneous depolarization of all excitable structures without any spreading response.

The most interesting finding with unstimulated tissue is the increase in respiration caused by ethanol, Cetavlon and PAD, all of which are known to be depolarizing agents. It supports the earlier suggestion[3] that depolarization may be the basis of the increased respiration in unstimulated tissue exposed to ethanol. This argument is weakened, however, by the absence of such effects in the presence of CPC. Dodecylpyridine chloride depolarizes[17] and one would accordingly expect CPC to act in the same way. On the other hand, there are exceptions to the general rule that quaternary ammonium compounds have a depolarizing effect.[12]

Atropine also gave results confirming McIlwain's[16] observations. The selective depression of respiration of stimulated tissue by PAD, and also by Cetavlon, constitutes further evidence for the view that the acetylcholine system is involved in the response to stimulation. It also supports the notion that inability to penetrate to the site of action is the reason for inactivity of lipid-insoluble quaternary compounds.

Although the predicted effects were obtained with the lipid-soluble quaternary compounds, this is no unequivocal demonstration of specific action on any of the supposed components of the acetylcholine system. On the basis of analogy with other preparations, however, the results, especially those with PAD and atropine, seem most readily explainable in terms of Nachmansohn's theory. As regards the surface-active compounds, the main argument for a specific action of CPC and Cetavlon in nerve tissue is their marked synergism with ethanol and the absence of such synergism between ethanol and Triton. In the test for haemolysis, the effect of Triton was clearly increased by ethanol, whereas that of Cetavlon was only very slightly altered, thus suggesting that the synergism between ethanol and Cetavlon in brain slices is associated with a process specific to nerve tissue.

Block of conduction by ethanol and by PAD is associated with depolarization. The similarity of their effects on brain tissue is striking. The only differences observed were, firstly the active concentrations, PAD being approximately 4000 times more effective than ethanol, and secondly the difference in the duration of the increase of respiration in unstimulated tissue. The difference in effective concentrations does not prove or disprove specificity of inhibition since it is penetration to the site of action that is decisive.[12] However, no obstacles to the penetration of ethanol would be

anticipated. Especially in view of the synergism on the one hand, and the structural and chemical differences on the other, it does not seem unreasonable to suggest that PAD and ethanol act on the same system, the former in a specific, the latter in an unspecific manner. However, ethanol may act on some structure not directly connected with the acetylcholine system, whereas action on the conducting membranes appears highly likely on account of the synergism with the quaternary ammonium compounds and with atropine.

The fact that the response to stimulation is not completely abolished with either PAD or ethanol may indicate that specific and complete inhibition of the acetylcholine system does not occur, or that operation of the acetylcholine system is not obligatory for the response of the slices to stimulation. The significance of reactions in the residual systems in presence of high concentrations of inhibitors is, however, very difficult to evaluate.

The findings with intact animals indicate that the observed synergism between ethanol and the quaternary ammonium compounds in the brain slices may have relevance for an understanding of alcohol intoxication. Ethanol might be thought to facilitate penetration of PAD in the same way as Cetavlon was more easily absorbed in intact animals when injected together with ethanol. However, the work of Wilson[13] suggests that PAD by itself is readily able to penetrate into brain tissue. Thus the results with the intact animals may reflect the same phenomena as were observed in the brain tissue.

The main conclusions of the present work are thus as follows. Firstly, lipid-soluble quaternary ammonium compounds selectively depress the respiration of brain tissue stimulated electrically as compared with that of unstimulated tissue. This is consistent with the theory that the acetylcholine system is necessary for the functioning of excitable tissue. Secondly, the synergism with ethanol is also restricted to the stimulated preparation. This suggests that ethanol may act directly on processes linked with the excitation cycle of conducting membranes.

Acknowledgement—The author wishes to thank Dr. Johan Järnefelt for stimulating discussions during the course of this work, and Drs. Heikki Suomalainen and Olof Forsander for their helpful criticism of the manuscript. He is also much indebted to Mrs. Kaija Salmela for her able technical assistance.

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