The Effect of Perchlorate Ion and Some Pharmacological Agents on the Sensitivity of the Frog (*Rana pipiens*) Rectus abdominis to Acetylcholine[1]

The frog rectus abdominis muscle preparation has been well established as a dependable and simple tool for acetylcholine assay in vitro[2], and has enjoyed wide use with the recent increasing interest in brain acetylcholine levels[3–7]. Extracts of brain may sensitize the frog rectus abdominis to acetylcholine after the intrinsic acetylcholine has been destroyed[7]. This sensitization has been observed with a variety of extraction procedures, and it is customary in bioassays to dilute the standard acetylcholine solutions in a brain extract which has been boiled in alkali and neutralized in order to control against this sensitization[6–9].

Various agents have been used to enhance the sensitivity of the frog rectus abdominis to acetylcholine. Physostigmine (eserine) sulphate (2 mg/l) is generally used for this purpose. However, neostigmine (Prostigmine) and tetraethylpyrophosphate (TEPP) have been suggested as alternates[10]. Additional sensitization has been achieved in preparations fully sensitized to a cholinesterase inhibitor with choline and potassium[8], and acetone[11,12]. On the other hand, atropine has been claimed to have no effect[5], while hemicholinium desensitizes the non-eserinized preparation[13]. Such observations would indicate that sensitization of the frog rectus abdominis to acetylcholine is not specific to one group or type of compound. Furthermore, agents used in the animal experiment or in the extraction procedure may well influence the sensitivity of the preparation.

The present report is concerned with an assessment of the influence of some of these agents on the sensitivity and accuracy of the assay. Emphasis has been placed on muscarinic and atropinic agents, since these have been reported to alter brain acetylcholine levels consistently[5,6].

Methods. Modified frog Ringer solution with the following composition was used: 114 m*M* NaCl, 2 m*M* $CaCl_2$, 1 m*M* KCl, 2 m*M* sodium phosphate buffer at pH 7.3 and 4 m*M* dextrose.

Pure O_2 was bubbled through the solution continuously and 2 mg/l physostigmine salicylate were added to the solution after the preparation had been allowed to equilibrate in the bath for 90 min. The assay was begun 60–90 min after addition of physostigmine. All solutions including the bath solution were kept at room temperature. The bath volume was 5 ml. Isometric contractions were recorded with a transducer (Statham No. G7A-1-2500) and Heath potentiometric recorder.

At the beginning of each experiment, a dose response curve to acetylcholine was obtained with a minimum of 3 replicate responses to each of 3 doses. Responses were measured 2 min after adding the drug; 5 min and 3 washings proved sufficient for complete recovery. In subsequent tests, various doses of possible modifiers were added 90 sec before a standard dose of acetylcholine, and controls using all 3 doses of acetylcholine were randomly interspersed. Except where specific interactions between modifiers were sought, only one was studied in each experiment. In every case, the effect of the modifiers was completely reversible when exposure time was limited in this way. It was expressed by reference to the acetylcholine dose-response curve as the factor (potentiation ratio) by which the dose of acetylcholine must be reduced in the presence of a modifier to obtain the same response seen after a standard dose of acetylcholine in its absence.

Results. With the exception of bethanechol, the muscarinic agents tested produced significant potentiation of the response to acetylcholine at concentrations too low to cause contraction themselves (Table I). Elevated K^+ levels caused little effect, in contrast to previous reports in the literature[8], until concentrations were reached which produced a contracture in the absence of acetylcholine. Paraoxon produced no further sensitization, presumably because the cholinesterase was already completely inhibited by physostigmine. Atropine reduced the sensitivity in sufficient concentration; 1,4-*bis*-(hexamethylenimo)-2-butyne (BHB), a central anticholinergic agent[14,15], produced no significant change.

The potentiation observed with muscarinic agents was more marked when the assay was conducted in the presence of brain extracts which had been boiled with alkali to destroy intrinsic acetylcholine; these extracts also caused significant potentiation in the absence of added muscarinic agents.

In these experiments, brains were extracted with cold perchloric acid which was subsequently precipitated by neutralizing with potassium carbonate[6]. Since potassium perchlorate retains a significant, though small, solubility at 0 °C (0.75 g/100 ml; or $5.4 \cdot 10^{-2} M$)[16], the possibility was considered that either K^+ or ClO_4^- ions might be responsible for this potentiation. Little potentiation was observed with added potassium (Table I). Perchlorate ions, on the other hand, produced a marked sensitization to acetylcholine when present at a concentration above approximately $2 \cdot 10^{-4} M$ (Table II), a level which is grossly exceeded when reliance is placed on the low solubility of potassium perchlorate to remove perchlorate ion (see above). When perchloric acid extracts of brain were neutralized by the addition of amine ion exchange resins in the basic form, sensitization to acetylcholine by the inactivated extracts was considerably less than when perchlorate was removed by K_2CO_3, suggesting that more efficient removal of perchlorate ion is achieved in this way (Table III).

The greater potentiation observed with muscarinic agents in the presence of brain extracts can also be interpreted, at least in part, as an additive effect with perchlorate. As Table IV shows, the potentiation produced by arecoline and perchlorate together is more marked than that occurring with either alone; similar results were

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Table I. Potentiation of response of eserinized frog rectus to acetylcholine produced by various compounds in the presence and absence of a brain extract (equivalent to 75 mg brain in 5 ml bath) in which intrinsic acetylcholine has been destroyed by boiling in alkaline solution. Potentiation ratio is the factor by which the dose of acetylcholine must be reduced to obtain the same response seen after a standard dose of acetylcholine in the absence of added modifier and brain extract

Drug	Concentration (M)	Potentiation ratio	
		Drug only	Drug + brain extract
Oxotremorine sesquioxalate (M.W. 341.3)	–	1.00	1.59
	$2.0 \cdot 10^{-6}$	–	1.77
	$5.0 \cdot 10^{-6}$	–	1.88
	$1.0 \cdot 10^{-5}$	1.00	2.38
	$2.0 \cdot 10^{-5}$	1.28	–
	$3.0 \cdot 10^{-5}$	1.29	3.35
	$3.6 \cdot 10^{-5}$	1.63	–
	$4.0 \cdot 10^{-5}$	1.75	–
	$6.0 \cdot 10^{-5}$	2.29[a]	3.91[a]
Arecoline hydrobromide (M.W. 236.1)	–	1.00	2.05
	$5.0 \cdot 10^{-6}$	1.00	2.00
	$1.0 \cdot 10^{-5}$	1.00	2.19
	$2.0 \cdot 10^{-5}$	1.03	2.39
	$4.0 \cdot 10^{-5}$	1.04	–
	$1.0 \cdot 10^{-4}$	1.44	3.23
	$1.6 \cdot 10^{-4}$	1.35	–
Aceclidine hydrochloride (M.W. 205.7)	–	1.00	2.61
	$8.0 \cdot 10^{-6}$	1.04	2.91
	$4.0 \cdot 10^{-5}$	1.00	3.82
	$1.0 \cdot 10^{-4}$	1.34	–
	$1.4 \cdot 10^{-4}$	1.46[a]	–
Bethanechol chloride (M.W. 196.7)	–	1.00	1.62
	$4.0 \cdot 10^{-5}$	1.20	–
	$1.2 \cdot 10^{-4}$	1.07	–
	$4.0 \cdot 10^{-4}$	1.03	1.92
	$2.0 \cdot 10^{-3}$	0.93	–
Carbachol chloride (M.W. 182.7)	–	1.00	1.72
	$1.0 \cdot 10^{-7}$	1.00	1.83
	$2.0 \cdot 10^{-7}$	1.03	–
	$4.0 \cdot 10^{-7}$	1.17	2.17
	$8.0 \cdot 10^{-7}$	1.21	2.17
	$1.6 \cdot 10^{-6}$	1.61	3.42
	$2.0 \cdot 10^{-6}$	1.69	–
	$3.2 \cdot 10^{-6}$	2.14[a]	–
	$4.0 \cdot 10^{-6}$	2.69[a]	–
Paraoxon (M.W. 275.2)	–	1.00	–
	$2.0 \cdot 10^{-9}$	1.00	–
	$2.0 \cdot 10^{-8}$	1.05	–
	$1.0 \cdot 10^{-7}$	1.00	–
	$5.0 \cdot 10^{-7}$	1.00	–
	$1.0 \cdot 10^{-6}$	0.94	–
Potassium chloride (M.W. 74.6)	–	1.00	–
	$5.4 \cdot 10^{-3}$	1.00	–
	$1.1 \cdot 10^{-2}$	1.00	–
	$1.6 \cdot 10^{-2}$	1.17	–
	$2.1 \cdot 10^{-2}$	1.44[a]	–
Atropine sulphate (M.W. 676.8)	–	1.00	2.42
	$1.0 \cdot 10^{-6}$	1.00	–
	$1.0 \cdot 10^{-5}$	0.86	2.79
	$2.0 \cdot 10^{-5}$	0.85	–
	$5.0 \cdot 10^{-5}$	0.55	1.16
	$1.0 \cdot 10^{-4}$	0.00	–
BHB dihydrochloride (M.W. 321.3)	–	1.00	3.29
	$4.7 \cdot 10^{-6}$	1.00	3.36
	$2.3 \cdot 10^{-5}$	–	4.33
	$4.7 \cdot 10^{-5}$	1.08	4.73
	$9.4 \cdot 10^{-5}$	1.13	–

[a] Indicates threshold for contracture in the absence of acetylcholine.

Table II. Potentiation of eserinized frog rectus abdominis to acetylcholine by various concentrations of sodium perchlorate

Concentration (M) $NaClO_4$	No. of measurements	Potentiation ratio
$1.6 \cdot 10^{-5}$	2	1.0
$8 \cdot 10^{-5}$	1	1.0
$1.6 \cdot 10^{-4}$	2	1.19
$8 \cdot 10^{-4}$	2	1.71
$8 \cdot 10^{-3}$	3	5.32[a]

[a] Threshold concentration for contracture in the absence of acetylcholine.

Table III. Potentiating effects of perchloric acid extracts of rat brain in which intrinsic acetylcholine has been destroyed by boiling in alkaline solution. The acid extract was neutralized and 'excess' perchlorate was removed by titration to neutrality with the reagent indicated at 0 °C followed by centrifugation

Reagent used for ClO_4^- removal	No. of brains extracted	Mean potentiation ratio
K_2CO_3	11	2.15
AG 3-X4	3	1.33
Bio-Rex 5	2	1.84
Bio-Rex 9	3	1.47

Table IV. Potentiation ratios for acetylcholine on eserinized frog rectus abdominis in the presence of various combinations of perchlorate and arecoline concentrations

ClO_4^- concentration (M)	Arecoline concentration (M)		
	0	$4 \cdot 10^{-5}$	$1.6 \cdot 10^{-4}$
0	1.00	1.00	1.35
$2 \cdot 10^{-4}$	1.00	1.18	1.49
$6 \cdot 10^{-4}$	1.34	–	1.90

obtained with oxotremorine. Desensitization produced by atropine was shown to decrease in the presence of perchlorate.

Discussion. The results presented show that several muscarinic and related agents may alter the sensitivity of the frog rectus abdominis to acetylcholine.

Uniform distribution of a drug after its in vivo injection in average doses would not result in concentrations in brain extracts as high as those required to sensitize the frog rectus. Caution is required in interpreting assays if the agents are used in large amounts, or may be concentrated in brain tissue. This is particularly so when brains are extracted with perchloric acid, since it has been shown that sensitization is seen at lower concentrations, and is more marked in the presence of perchlorate ion.

The degree of sensitization produced by perchlorate alone is concentration-dependent in the range 10^{-4}–10^{-2} M, and a spontaneous contracture is elicited by perchlorate

at the upper limit of this range. Deliberate inclusion of perchlorate in a fixed concentration cannot be recommended as a way to enhance sensitivity, since its effects are variable and somewhat time-dependent. Complete removal is obviously desirable prior to assay if perchloric acid is used to extract tissues; precipitation as the potassium salt is less satisfactory in this respect than neutralization with a basic anion exchange resin.

Sensitization of the frog rectus abdominis by perchlorate is of some interest in itself. There is evidence that it is analogous to the potentiation of contraction by other foreign anions according to their position in the Hofmeister series[17]. Perchlorate has been shown to reduce chloride permeability and prolong the active state in frog sartorius muscle[18], and is roughly equivalent to thiocyanate in this respect. Other authors place perchlorate beyond thiocyanate in the lyotropic series[19].

In summary, perchloric acid is probably best avoided as an extracting agent prior to bioassay of acetylcholine because of the variable sensitization it produces particularly when other drugs may be present. When it is used for this purpose, its subsequent removal with an ion exchange resin appears desirable.

Zusammenfassung. Die Reaktion des mit Eserin vorbehandelten musculus rectus abdominis des Frosches auf Acetylcholin liess sich durch Oxotremorin ($2.0 \cdot 10^{-5} M$), Arecolin ($2.0 \cdot 10^{-5} M$), Aceclidin ($1.0 \cdot 10^{-4} M$) und Carbachol ($2.0 \cdot 10^{-7} M$) verstärken, während Atropin ($1.0 \cdot 10^{-5} M$) hemmend wirkte. Perchlorationen in Konzentrationen über $2 \cdot 10^{-4} M$ sensibilisierten den Froschmuskel erheblich und reduzierten die Grenzdosis, bei der eine Potenzierung durch Substanzen mit muskarinähnlicher Wirkung beobachtet wird. Alle beschriebenen Reaktionen liessen sich durch Gehirnextrakte verstärken, in denen zuvor Acetylcholin durch Kochen in alkalischer Lösung zerstört worden war.

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Increased Level of 5-Hydroxyindoleacetic Acid in Cerebrospinal Fluid from Infantile Hydrocephalus

Carlsson, Falck and Hillarp[1] have suggested that 5-hydroxytryptamine (5-HT), as well as noradrenalin and dopamine, are transmitters in the central nervous system. Bogdanski, Weissbach and Udenfriend[2] reported that 5-HT appears in the cerebrospinal fluid (CSF) of rats and dogs following administration of 5-hydroxytryptophan, the precursor of 5-HT. However, it has not been possible to show the presence of 5-HT in CSF under normal conditions using sensitive spectrophotofluorometric methods. On the other hand, 5-hydroxyindoleacetic acid (5-HIAA) can be fairly easily determined in CSF. Ashcroft and Sharman[3] and Sharman[4] showed that the level of this acid is increased in CSF from hydrocephalic patients. Their materials were not used for diagnostic purposes.

The clinical diagnosis of hydrocephalus is fairly easy in advanced cases. However, with the new surgical method (ventriculo-venous-shunts) in which the results are uniformly better[5], the demand for early diagnosis, and hence earlier treatment, has increased. The early treatment produces better results.

The conventional diagnostic methods using pneumoencephalography and ventriculography carry a certain risk. They are not suitable for screening purposes. A simple laboratory test has so far not been evaluated, and our intention has been to produce a diagnostic method based upon determination of acidic monoamine metabolites in CSF. We have investigated hydrocephalic children and children suspected of hydrocephalus. Some preliminary results were presented at the Meeting of Scandinavian Neurologists in 1964[6].

Both groups have been subjected to a careful clinical investigation using other available methods, e.g. encephalography, ventriculography and echo-encephalography[7], in order to establish the correct diagnosis. Only children up to 1 year of age have been included in this report, i.e. cases of the so-called infantile hydrocephalus. 75 children were investigated. 39 of them had hydrocephalus and were operated upon. The remaining 36 were suspected of having the disease but further investigations could not verify the diagnosis.

The values of 5-HIAA in CSF of the 75 children are tabulated in the Table. Group II consists of non-hydrocephalic children undergoing investigations for various neurological conditions. It is seen that the level of 5-HIAA in the hydrocephalic group is in all cases above 0.09 µg/ml,

5-HIAA	Group I 'hydrocephalic'	Group II 'non-hydrocephalic'
$\leqq 0.08$	0	23
0.09–0.10	1	10
0.11–0.14	7	3
$\geqq 0.15$	31	0
Total	39	36

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