In the dichlorobenzoic acids myotonia occurred with the four acids having one or two chlorine atoms *ortho* to the carboxyl group, but not with the two having no *ortho* substituent. Also, these last two were of rather higher toxicity than the others.

In the tri- and probably the tetrachlorobenzoic acids, myotonia did not occur when all the chlorine atoms were adjacent, but did when any two were separated by hydrogen. Two of the trichlorobenzoic

TABLE 1. MOUSE TOXICITY AND MYOTONIC ACTION OF POLYCHLOROBENZOIC ACIDS

Acid	M.P. °C*	Mouse s/c LD50 mg/kg	Myotonia
2,6-dichloro-	140–6	1500	+ve
2,5-dichloro-	154	1200	+ve
2,4-dichloro-	164	1200	+ve
2,3-dichloro-	164–7	900	+ve
3,5-dichloro-	183–8	250	−ve
3,4-dichloro-	204–8	400	−ve
2,3,6-trichloro-	124–8	1500	+ve
2,3,5-trichloro-	163	300	+ve
2,4,6-trichloro-	163–4	1200	+ve
2,4,5-trichloro-	163–8	300	+ve
2,3,4-trichloro-	187–8	300	−ve
3,4,5-trichloro-	203–10	250	−ve
2,3,4,6-tetrachloro-	131–2	—	+ve together
2,3,5,6-tetrachloro-	178–9	—	+ve together
2,3,4,5-tetrachloro-	186–94	—	−ve

* Range of available separate values

acids were rather less toxic than the other four, and these were the ones in which both *ortho* positions carried chlorine atoms.

This effect of *ortho* substitution on the toxicity may be compared with the statement[3] that *ortho* substitution of benzoic acids always reduces conjugation *in vivo*.

As an empirical observation in all three series, the isomers of highest melting point were those which did not produce myotonia.

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Interference by physostigmine and serotonin in the colorimetric determination of acetylcholine

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IN THE course of a study dealing with acetylcholine metabolism in the spinal cord, physostigmine (eserine) was orginally used routinely as inhibitor of acetylcholinesterase (AChE; 3.1.1.7.).* When

* *Report of the Commission on Enzymes*, Pergamon Press, London (1961).

acetylcholine was determined by the colorimetric method of Hestrin,[1] physostigmine was found to interfere, giving an intense brown color similar to the ferric hydroxamate formed from acetylcholine. The reaction of metal ions and hydroxamate with a great number of compounds has been studied,[1-3] but the behavior of physostigmine with this reagent has not been reported.

Although physostigmine is routinely used as inhibitor of AChE, the usual methods of application of Hestrin's acetylcholine determination do not refer to this interference. Under the usual experimental conditions, the concentration of acetylcholine generally exceeds the concentration of physostigmine by two to three orders of magnitude, and interference by physostigmine would not be detectable. However, when considerably higher concentrations of this inhibitor are used, such as in studies dealing with the carbamylation of AChE by physostigmine and related compounds,[4-6] the present observations may become important. The reaction of physostigmine with alkaline hydroxylamine and ferric salts was studied in greater detail; similar studies were also carried out with serotonin (5-hydroxytryptamine), which is a natural inhibitor of AChE[7, 8] and related compounds.

The following reagents were used: acetylcholine chloride (Merck); physostigmine sulfate (Calbiochem, C grade); physostigmine salicylate (Inland Alkaloid, USP); Prostigmine (neostigmine) methylsulfate (Roche); L-tryptophan (Mann, M.A.); DL-5-hydroxytryptophan H_2O (Calbiochem, A grade); 5-hydroxytryptamine hydrogen oxalate (Regis); 5-methoxytryptamine (Regis); hydroxylamine hydrochloride (Fisher, reagent grade). All other reagents were reagent grade. For the determination of acetylcholine and interfering compounds, the colorimetric method of Hestrin[1] was used without modifications. Graded levels of acetylcholine solutions and physostigmine or other reactants were diluted with water to a total volume of 1·50 ml. Equal parts of 2 M hydroxylamine chloride and 3·5 M NaOH were mixed previous to the assay, and 2·0 ml of this mixture was added to the test solution. After standing for 10 min at room temperature the mixture was acidified by the addition of 1·0 ml of HCl 1 : 3, and color was developed by the addition of 1·0 ml of 0·37 M ferric chloride. Color was read at 540 mμ in a Bausch and Lomb "Spectronic 20" spectrophotometer. Absorption spectra of the developed color were obtained in a Zeiss-Gilford spectrophotometer, with the complete system as blank and water replacing the test substance, in cells with 1·0 cm light path at 25° and pH 1·0.

The effect of physostigmine sulfate in the colorimetric determination of acetylcholine is shown in Fig. 1. Physostigmine produces an intense brown color, similar in hue at 540 mμ to the ferric hydroxamate obtained from acetylcholine. It can be seen that the addition of physostigmine to the reaction mixture enhanced the color of the solution. The slope of the standard curve obtained with acetylcholine is not changed by the addition of physostigmine sulfate. Analogous results were obtained when physostigmine salicylate was used. (It should be noted that physostigmine sulfate has the composition $(C_{15}H_{21}N_3O_2)_2\,H_2SO_4$ and therefore presents double the physostigmine concentration present in physostigmine salicylate, in equimolar solutions; for the sake of simplicity, though, both neostigmine and physostigmine concentrations are expressed in terms of molar solution in Fig. 1). On the other hand, neostigmine does not interfere in the colorimetric assay, as has already been shown by Hestrin, and does not produce a color when tested alone. For this reason, neostigmine should be preferred as inhibitor of AChE, when the assay procedure used involves determination of acetylcholine by the colorimetric method.

When high levels of physostigmine are used as AChE inhibitor, care must be taken to include suitable corrections for the color produced by physostigmine in appropriate blank solutions. It should be pointed out that even corrections by means of blanks containing appropriate concentrations of physostigmine need not always be valid, and would hold true only if the concentration of unbound physostigmine were constant in all components of the assay system. It is known that physostigmine itself is hydrolyzed at a slow rate by AChE,[9, 10] and it may be difficult to have a precise control over binding and catabolism of physostigmine in a complex test system.

Attempts were made to destroy physostigmine selectively previous to assay of acetylcholine. A great many conditions of alkaline treatment were tried without success. Acetylcholine is characterized by its alkali-lability; physostigmine is much more resistant to alkaline treatment than is acetylcholine, but it, too, is destroyed by alkaline treatment, as has been previously shown,[10] and confirmed by the present author. When treated singly, acetylcholine was destroyed completely by incubating for 1 hr at 25°, or by boiling for 1 min in 0·0025 N NaOH. Under these conditions 24 and 34% of the chromogen derived from physostigmine was destroyed. However, under experimental conditions in which even physostigmine alone would be completely destroyed, acetylcholine and physostigmine interact, forming an undetermined stable chromogen which gives an intense reaction under the usual conditions

of assay. It is not known whether a similar protective effect would be obtained with other natural mixtures containing acetylcholine, when the mixture is boiled in alkaline solution. If this were true, characterization of acetylcholine in a physiologically active material should include bioassay after boiling in alkali, to rule out false identifications by means of the colorimetric hydroxamate method.

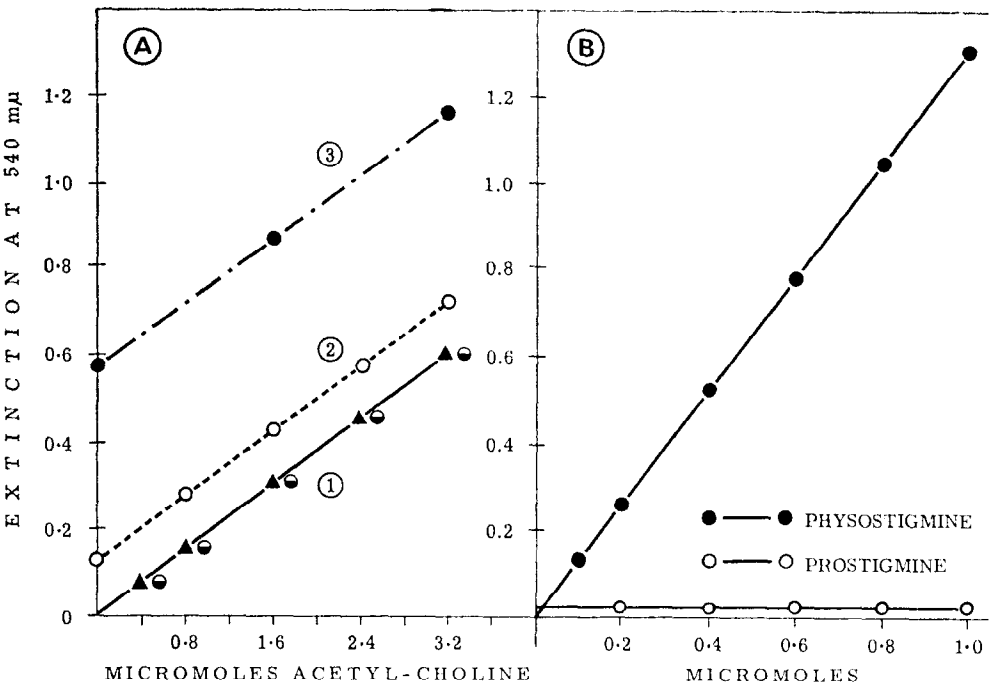

FIG. 1. A: Effect of physostigmine sulfate and neostigmine methylsulfate on color produced by acetylcholine. Curve 1: ▲——▲ acetylcholine; ◒——◒ acetylcholine + 0·1 μmole neostigmine Curve 2: acetylcholine + 0·1 μmole physostigmine. Curve 3: acetylcholine + 0·4 μmole physostigmine. B: Reaction of physostigmine sulfate and neostigmine methylsulfate with hydroxylamine and ferric chloride.

Contrary to what occurrred in alkaline solution, separation of acetylcholine and physostigmine could be carried out successfully in acid solution. By heating for 30 min in boiling water bath in 0·05 M HCl, 31% of the acetylcholine was destroyed, whereas 100% destruction of acetylcholine was obtained when 0·5 M HCl was used. Even the high concentration of HCl did not affect the chromogen obtained from physostigmine; no protective interaction took place between physostigmine and acetylcholine in acid solution. Acid treatment thus permits simultaneous assay of acetylcholine and physostigmine. The two reagents can also be separated by chromatography or solvent extraction, physostigmine passing into ether, for example, from slightly alkaline aqueous solutions.

While interference in the colorimetric determination of acetylcholine by artificial inhibitors can be avoided by proper selection of reagents and appropriate experimental conditions, interference by compounds naturally present in homogenates containing acetylcholine must also be kept in mind. Such a compound is, for example, serotonin (5-hydroxytryptamine, 5-HT), which is known to inhibit AChE.[7, 8] When serotonin and its parents compound (5-hydroxytryptophan, 5-HTP) were tested in the colorimetric hydroxamate method of Hestrin, an intense brown color was formed, similar to the color produced by acetylcholine. The interference of 5-HT and 5-HTP in the colorimetric assay of acetylcholine is shown in Fig. 2. The greatest color intensity was shown by 5-HT. While color developed instantly with acetylcholine, the reaction was slower with 5-HT and 5-HTP. The intensity of color

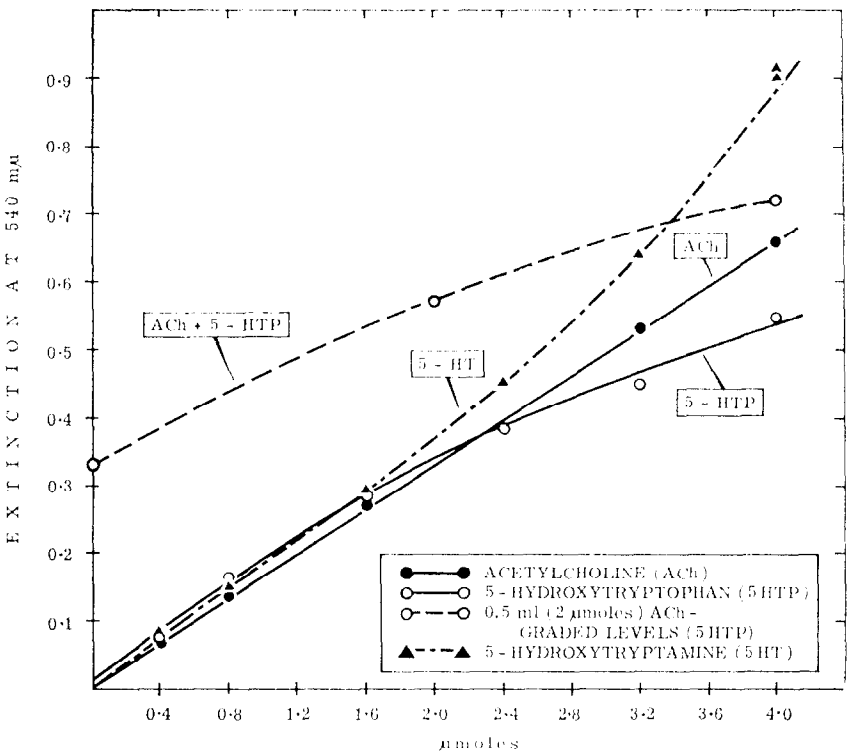

FIG. 2. Reaction of 5-HT and 5-HTP with hydroxylamine and ferric chloride.

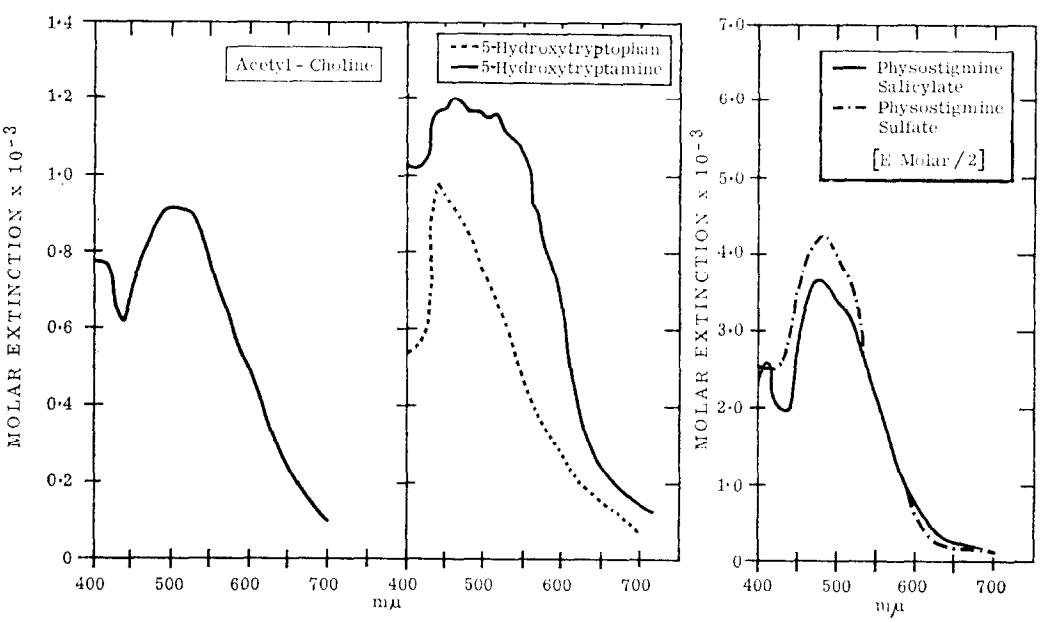

FIG. 3. Absorption spectra of color formed with alkaline hydroxylamine and ferric chloride. Color formed from 5-HT and 5-HTP was read after standing at 25° for 30 min.

formed for 5-HT increased at a steep rate during the first 8 min at 25°, and reached a plateau after 15 min. The absorption spectra of the colored compounds obtained by reacting alkaline hydroxylamine and ferric salts with physostigmine, 5-HT, and 5-HTP are shown in Fig. 3, and the spectrum obtained with acetylcholine is included for comparison.

It is interesting to note that neither tryptophan nor 5-methoxytryptamine (O-methylserotonin) formed a colored substance under the present experimental condition, whereas both 5-HT and 5-HTP formed this color. This indicates that a structural requirement for color development by this reaction is the presence of a free phenolic OH-group in an indole ring system. It would be interesting to test serotonin isomers, with the hydroxy-group in different positions. Physostigmine contains such a hydroxy-indole structure. This is absent in neostigmine, a compound that forms no color. The structure of the chromogen is not yet known; it is likely that serotonin and its structural analogs form a quinone or semiquinone-like compound, which would react with hydroxylamine to form an oxime, which in turn could react with iron salts, forming a colored complex or chelate. It should be remembered that the rate of color development proceeds much more slowly with 5-HT than with acetylcholine or other reagents forming true hydroxamates; this indicates that the mechanism of reaction is probably different in the two cases. It is interesting to note that 5-methoxytryptamine, which does not form a color in the present method, has been shown to be considerably less active as inhibitor of AChE than is 5-HT;[11] whether this constitutes more than coincidence remains to be seen.

The "hydroxamate" color was explored as an assay procedure for the determination of physostigmine, and proved to be a simple and sensitive method in which a linear relationship obtained between the concentration of physostigmine and the absorbance. Color was formed by applying Hestrin's method without further modification, and was read at the absorption maximum 490 mμ; the curve obtained was linear over a wide range at this wavelength or other convenient wavelengths in this region. During color development, a pink color was formed after the addition of hydrochloric acid, which probably corresponds to the formation of rubreserine. This color was not always proportional to the physostigmine concentration and was not investigated further.The controlled transformation of physostigmine to rubreserine has served as a basis for colorimetric assay for physostigmine.[10, 12, 13]

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