which is capable of spreading. The spreading test may also be applied in certain cases to the detection of solid contaminants, such as detergents which are leached by solvents. The recent report of silicones present in disposable syringes (10) suggests another possible application. Silicones are surface active; if spread on water, as little as 0.01 mg. can easily be determined (11).

Although the spreading method is very sensitive, it is limited to those impurities which are not very volatile and which have some affinity for the surface. Included in this category are surfactants, lipids, most polymers, and some other materials such as steroids and antibiotics. The method is incapable of detecting, for example, the presence of low boiling homologs or isomers in hexane. Therefore it should not be considered a substitute for other methods of checking purity. Nevertheless, if it is necessary to determine trace quantities of surface-active impurities, the spreading method represents a convenient and efficient tool.

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Anticholinesterase Activity of Some Degradation Products of Physostigmine

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Abstract ☐ The anticholinesterase activities of four degradation products of physostigmine have been determined *in vitro* using biochemical and biological assay techniques and *in vivo* by means of the rat chromodacryorrhea response. The degradation products are at least 100 times less active than the parent compound as anticholinesterase agents. Eseroline, rubreserine, and eserine blue are effective in high concentrations against the cholinesterase of horse and rabbit plasma but are much less active against the red blood cell cholinesterase; they exert no inhibitory action on the two human cholinesterase enzymes. *In vivo*, the degradation products are at least 1000 times less active than physostigmine. Lack of coloration of solutions of physostigmine does not necessarily indicate full anticholinesterase activity because a colorless product of hydrolysis, eseroline, possesses little or no anticholinesterase activity.

Keyphrases ☐ Physostigmine—degradation products ☐ Anticholinesterase activity—physostigmine degradation products ☐ Biological assay—physostigmine degradation products ☐ *In vitro* assay—physostigmine degradation products activity

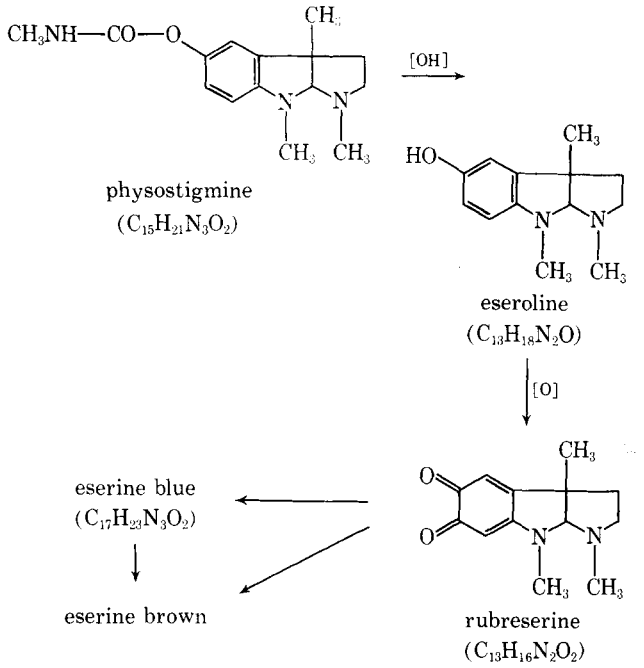
When physostigmine in solution undergoes decomposition, a red-colored compound is formed which turns blue on further decomposition (1). Hydrolysis which initially removes the urethane grouping and produces eseroline, a colorless compound (Scheme 1), is followed by oxidation to yield rubreserine, a red material, which is converted into eserine blue or eserine brown. The molecular weight of eserine blue exceeds that of rubreserine and it has been suggested that condensation of rubreserine with other physostigmine degradation products occurs at this stage in the reaction. The end product of the degradation by heat or by exposure to oxygen and alkali is eserine brown.

The anticholinesterase activity of these degradation products has been investigated. Their biological activity has particular relevance as solutions of physostigmine are used in ophthalmology. Furthermore, in the preparation of eye drops sterilization is effected by filtration or by autoclaving the solution at 98 to 100° for 30 min. (BPC, Supplement, 1966) or by steam sterilization at 121°. (USP XVII, 1965). This heating process may result in decomposition of the physostigmine, with possible loss of activity. A preliminary note concerning this work has been published (2).

METHODS

Warburg Manometric Technique—The anticholinesterase activity of the different compounds was compared by this technique using horse serum (0.5 ml.) as the source of cholinesterase and acetylcholine (0.5 ml.) at a final concentration of 0.0138 *M* (3) as the substrate. The pH value of the incubation mixture was controlled by adding 1.5 ml. of 0.04 *M* sodium bicarbonate (adjusted to pH 7.6 by the addition of hydrochloric acid) and each compound under test was added to 0.5 ml. of this bicarbonate solution before addition to the incubation flask. The total volume of fluid in each flask was 3 ml. After gassing with a mixture of 95% nitrogen and 5% carbon dioxide for 10 min., the flasks were incubated at 37° and the manometers read at 10-min. intervals for 30 min. Values in the text are the mean of three experiments. Differences in percentage inhibition greater than 15% are significant ($p = 0.05$).

Biological Assay Technique—Horse serum (0.1 ml.) was used as the source of cholinesterase, with acetylcholine as the substrate (2 ml. of 5 mcg./ml.). Each compound under test was dissolved in 2



Scheme I—*The relationship between physostigmine and some of its degradation products.*

ml. of 0.04 *M* sodium bicarbonate and added to the incubation mixture. Incubation was carried out at 37° for 30 min., during which time all of the acetylcholine was hydrolyzed when no anticholinesterase was present. In the presence of an inhibitor, the residual acetylcholine was assayed biologically on three different preparations. The isolated colon and uterus of a rat were set up in de Jalon's solution at 32° while the ileum of a guinea pig was suspended in Tyrode's solution also at 32°. Contractions were recorded isotonically on a Grass polygraph. Assays were carried out using the 2 × 2 dose schedule. The amounts of anticholinesterase and horse serum used had no effect on any of these assay preparations. Values in the text are the mean results from the three preparations. Differences in percentage inhibition greater than 15% are significant ($p = 0.05$).

Anticholinesterase Activity Using Plasma and Red Blood Cells—Blood was collected from rabbits, man, and horses into heparinized syringes. After centrifugation to obtain the plasma, the red cells were washed three times with 0.9% (w/v) saline and then haemolyzed in a volume of distilled water corresponding to the initial volume of plasma. The anticholinesterase activities of the compounds under test were then compared using the biological assay techniques, as described previously.

Chromodacryorrhea Test in Rats—Male rats (100–200 g.) were first injected subcutaneously with acetylcholine (200 mcg./100 g. rat) and those producing red tears within 2 min. were used (4). The anticholinesterase activities of the test compounds were then evaluated by injecting them intraperitoneally 30 min. before the subcutaneous injection of a smaller dose of acetylcholine (20 mcg./100 g. rat), and examining for the presence of red tears at 2-min. intervals for 14 min.

Degradation Products of Physostigmine—Samples of eseroline, rubreserine, eserine blue, and eserine brown were supplied by Dr. G. A. Smith, Department of Pharmacy, Heriot-Watt University, Edinburgh, Scotland. They were dissolved in freshly distilled water and the solutions were stored at 4° until required.

RESULTS

Anticholinesterase Activity Determined Manometrically—Concentrations of physostigmine as low as 10^{-8} *M* were effective in inhibiting the cholinesterase of horse serum and higher concentrations had correspondingly greater activities (Fig. 2). All the degradation products tested were less active than the parent compound and activities of these products at concentrations of 5×10^{-5} *M* only

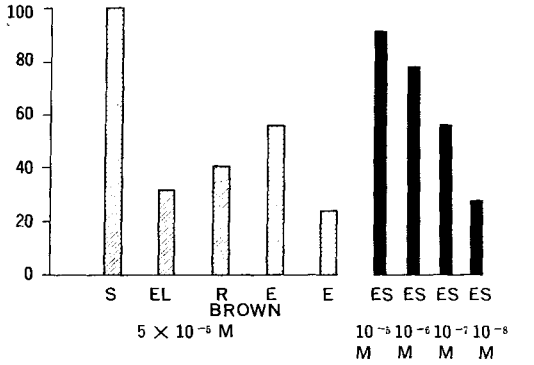


Figure 1—*The anticholinesterase activities of physostigmine (ES), eseroline (EL), rubreserine (R), eserine blue (E Blue), and eserine brown (E Brown), determined manometrically using horse serum as the source of cholinesterase. The ordinate shows percentage inhibition of cholinesterase with reference to 5 × 10⁻⁵ M physostigmine as 100%. The effects of graded concentrations of physostigmine are also shown for comparison.*

are recorded. Eserine blue was the most active of these products and at this concentration produced 52% inhibition of the cholinesterase, comparable with that produced by physostigmine at 10^{-7} *M* (Fig. 1). Thus, eserine blue is at least 500 times less active as an anticholinesterase agent, and eseroline is about 5000 times less active in this test.

Anticholinesterase Activity Determined Biologically—Concentrations of physostigmine as low as 10^{-7} *M* were effective in reducing the cholinesterase activity of horse serum and higher concentrations exerted greater activity (Fig. 2). All the degradation products were less active than the parent compound which reduced cholinesterase activity to 54% of control at 10^{-7} *M*. The most active of these products, eserine blue, is about 100 times less active than physostigmine in this test.

Inhibition of Cholinesterase of Plasma and Red Blood Cells—Concentrations of physostigmine greater than 10^{-5} *M* completely inhibited both plasma and red cell cholinesterase of rabbit, horse, and man. Comparisons of the activities of the degradation products of physostigmine are shown in Fig. 3 where only concentrations of 5×10^{-5} *M* are recorded. Eserine blue, the most active of the degradation products, inhibited the pseudo-cholinesterase of rabbit plasma by about 50%, but it was almost inactive against the true cholinesterase of the red cells; against the cholinesterase of horse plasma, it exerted about 60% inhibition (as it did against horse serum, Figs. 1 and 2) whereas it was only slightly less active against the cholinesterase of horse red cells; however, it was inactive against the cholinesterase enzyme of both human plasma and red cells. This difference

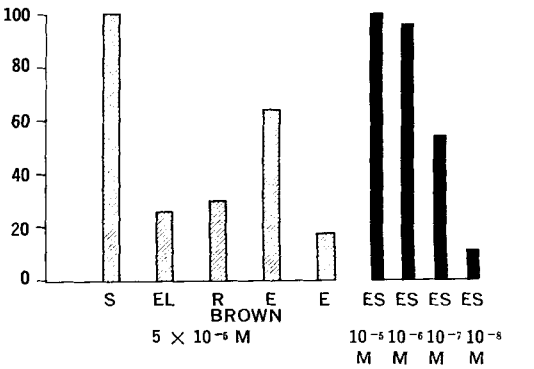


Figure 2—*The anticholinesterase activities of physostigmine (ES), eseroline (EL), rubreserine (R), eserine blue (E Blue), and esrine brown (E Brown), determined biologically using horse serum as the source of cholinesterase. The ordinate shows percentage inhibition of cholinesterase with reference to 5 × 10⁻⁵ M physostigmine as 100%. The effects of graded concentrations of physostigmine are also shown for comparison.*

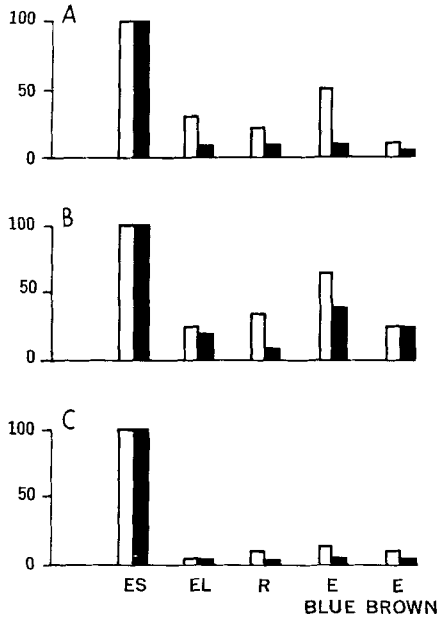


Figure 3—*The anticholinesterase activities of physostigmine (ES), eseroline (EL), rubreserine (R), eserine blue (E Blue), and eserine brown (E Brown) determined biologically using plasma (open columns) and red blood cells (closed columns) as sources of cholinesterase. Activities recorded on the ordinate are percentages of those obtained with 5 × 10⁻⁵ M physostigmine. All compounds were tested at concentrations of 5 × 10⁻⁵ M. A = rabbit, B = horse, C = man.*

in activity of eserine blue against the cholinesterases of all three species contrasts markedly with that of physostigmine, which was equally active against the true and pseudocholinesterases, even at concentrations of 10^{-8} *M*. The other degradation products of physostigmine were less active than eserine blue; with the rabbit and horse enzymes, they had greater effects on the plasma cholinesterase, but with the human enzymes, all were without significant effect at concentrations of 5×10^{-5} *M*.

Rat Chromodacryorrhea Response—Physostigmine was active at doses of 0.1 mg./kg., red tears being produced within 2 min. of the injection of acetylcholine. However, eserine blue, rubreserine, and eseroline were about 1000 times less active than physostigmine in this test. Eserine brown was inactive at all dose levels tested.

DISCUSSION

The results show that the degradation products of physostigmine used in the present work are all much less active as anticholinesterase agents than the parent compound. These products lack the urethane grouping, but it is of interest that when oxidation occurs the anticholinesterase activity first increases and then decreases. Thus, eserine blue is more active than rubreserine or eseroline and under certain circumstances may exert an activity equivalent to about 1% of the parent compound. This result agrees with that of Ellis *et al.* (5) who showed that eserine blue was more active than rubreserine as an anticholinesterase agent. However, these workers were unable to find any anticholinesterase activity in eseroline or eserine brown.

The results using the standard Warburg technique agree well with those involving biological assay of the residual acetylcholine, when a standard enzyme preparation such as horse serum is used. When the two cholinesterases of horse blood are used as the source of the enzyme and comparisons are made of the anticholinesterase activities of the degradation products, rubreserine and eserine blue have a greater inhibiting effect on the plasma cholinesterase. With rabbit blood, almost no activity has been found at the high concentrations tested on the red cell enzyme although results with the plasma enzyme have corresponded well with those obtained using the horse enzyme. Both human plasma enzyme and red cell cholinesterase are not inhibited to any great extent by these degradation products when used at concentrations of 5×10^{-5} *M;* however, physostigmine is equally active against both enzymes in much lower concentrations (6, 7). It is most likely that species differences of plasma cholinesterase (8–10) play a role in the different degrees of inhibition of cholinesterase produced by the degradation compounds.

From the clinical point of view, only physostigmine therefore exerts an anticholinesterase activity, and this is supported by the results of the *in vivo* test where the red tear response involving protection of the injected acetylcholine from both true and pseudocholinesterases shows that all the degradation compounds (including eserine blue) are 1000 times less active than physostigmine. The similar activities of the degradation compounds *in vivo* may be due to the fact that male rats were used in this test and male rat serum has been shown to contain much lower levels of pseudo-cholinesterase than does the serum of female rats (11, 12).

Since all ophthalmic solutions (BPC and USP) are now required to be sterile and physostigmine eye drops may be sterilized by heat, the formation of relatively inactive decomposition products (one of which is colorless) must be taken into account. These intermediate products have no therapeutic value and so the appearance of a pink color is not the only guide to loss of activity of the solution because a colorless solution may also be inactive.

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