would certainly lead to inhibition of histamine release induced by both antigen and A 23187 since both evoke histamine release which is dependent on an intact mechanism for ATP synthesis [11, 20].

Our results are consistent with the hypothesis that intracellular cyclic AMP regulates the calcium gating mechanism which is supposed to control histamine secretion. Measurements of intracellular cyclic AMP and calcium uptake are needed to investigate this further.

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Trialkyl phosphates and phosphorothiolates—Lack of hydrophobic interaction with acetylcholinesterase

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Several publications from this laboratory [1–3] and Russian laboratories [reviewed in Ref. 4] have indicated that trialkyl phosphates, phosphorothiolates and phosphonothiolates are potent anticholinesterases when at least one alkyl chain is of adequate length. This effect has been attributed to hydrophobic interaction of the inhibitor with the enzyme, resulting in high affinity in the reversible (K_d) step, despite the anticipated low reactivity in the irreversible (k_2) step; the process may be depicted as:

$$(R'O)_2P(O)XR + EOH \overset{K_d}{\rightleftharpoons} (R'O)_2P(O)XR \cdot EOH \xrightarrow{k_2} (R'O)_2P(O)OE + RXH$$

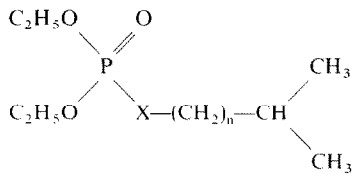
where X = *O* or *S*, EOH represents the enzyme, $K_d (= k_{-1}/k_1)$ is the dissociation constant for the first (binding) step and k_2 is the phosphorylation rate constant.

Recently, I. B. Wilson* raised doubts concerning the kinetic feasibility of the k_2 step in view of the poor leaving-group character of *SR* and *OR* moieties, while Gumbman and Williams [5] have reported the production of potent impurities during the synthesis of triethyl phosphate. Subsequently Gazzard *et al.* [6] found that the synthetic route used by Bracha and O'Brien [1–3], starting with diethyl phosphorochloridate, led to the production of an alkali-labile impurity which accounted for all the anticholinesterase activity in the case of the one compound they studied, namely diethyl *S*-*n*-propyl phosphorothiolate. We did not consider that this single finding, using the *n*-propyl derivative, was sufficient to call into question the hypotheses arising out of the earlier work, unless supported by similar results for other members of the class.

In view of the evidence discussed above, we have now undertaken a re-examination of the activities of these compounds and report here our findings for a series of phos-

* Personal communication.

phates having structure I and of phosphorothiolates having structure II:



I. X = *O*; II, X = *S*.

Small quantities (0·1 to 0·2 ml) of *O,O*-diethyl *O*-(ω-isopropyl) alkyl phosphates (I) and *O,O*-diethyl *S*-(ω-isopropyl) alkyl phosphorothiolates (II), previously synthesized in these laboratories [3], were shaken for 5 min with 40–50 ml of 0·1 N aqueous NaOH at ambient temperature (23°). Solutions were then neutralized by titration with diluted HCl. Each organophosphorus compound was then extracted from aqueous solution using three or four 10-ml portions of $CHCl_3$, following which the $CHCl_3$ was removed by evaporation under reduced pressure over a hot water bath. Infrared spectra of the resulting residues and the untreated compounds were determined using a Perkin–Elmer Infracord spectrophotometer.

Inhibitory potencies were determined in 0·1 M phosphate buffer at pH 7 and 25° using bovine erythrocyte acetylcholinesterase (acetylcholine acetyl hydrolase, EC 3.1.1.7, from Winthrop Laboratories). Determinations were carried out in the presence of a chromogenic substrate (1 mM *p*-nitrophenyl acetate) according to the method described previously [7, 8], using a Durrum model 110 stopped-flow spectrophotometer coupled to a Tektronix 5031 storage oscilloscope to follow the change in absorbance at 400 nm.

The infrared spectra of all the compounds studied (n = 1 to n = 4 for both structures I and II) were similar before and after treatment with 0·1 N aqueous NaOH. In particular, the ratios of absorbance peaks due to the *P = O* and *C–H* stretching vibrations at about 7·9 and 3·3 μ respectively were not appreciably altered by the treatment, indicating that the compounds were resistant to hydrolysis under the conditions used. None of the NaOH-treated compounds showed any ability to phosphorylate the enzyme; reaction profiles were linear, in contrast to the previously reported progressive phosphorylation of the enzyme by compounds of this class [1–3, 7].

Dissociation constants were calculated using the equation [7]

$$K_d = \frac{K_m[PX]}{(K_m + [S])[(v_c/v_0) - 1]} \quad (1)$$

where K_m is the Michaelis constant for the chromogenic substrate (K_m = 2·04 mM in this system), [PX] represents the concentration of organophosphorus compound, [S] the concentration of substrate, v_c the initial velocity of the reaction carried out in the absence of PX, and v_0 the initial velocity of the reaction in the presence of PX. Table 1 shows that the NaOH-treated compounds appear to act as very weak reversible inhibitors, K_d values being in the region of 10^{-2} M and showing no significant general correlation with alkyl chain length.

Our previous work using these compounds [1–3, 7] suggested that simple trialkyl phosphates and phosphorothiolates were potent and progressive inhibitors, in which the poor leaving-group character (leading to a low k_2) was compensated for by excellent affinity (leading to a low K_d), this being caused by hydrophobic interaction with the enzyme.

Table 1. Apparent dissociation constants for the interaction between acetylcholinesterase and alkali-treated diethyl alkyl phosphates (I) and phosphorothiolates (II) at 25° and pH 7

Series	n	K_d* (mM)	No. of determinations
I	4	11·93 (±5·43)	4
Phosphates	3	8·72 (±1·53)	4
(X = *O*)	2	8·85 (±1·74)	4
	1	10·51 (±2·11)	4
II	4	3·64 (±0·75)	5
Phosphorothiolates	3	7·28 (±0·54)	3
(X = *S*)	2	6·22 (±1·18)	5
	1	9·18 (±4·03)	4

* Standard deviations are shown in parentheses.

The revised findings show: (1) that these compounds are weak and nonprogressive inhibitors and that the earlier conclusions were based upon the use of impure samples; and (2) that there is little variation in the inhibitory activity of these compounds in spite of the range of their hydrophobic character; consequently hydrophobic interactions do not appear to be involved in their inhibition of acetylcholinesterase.

The values listed in Table 1 are very high and show that these are weak reversible inhibitors. These results should be regarded as upper limits, because of the possibility that this modest level of activity could be due to chloroform-extractable impurites. However, if the active impurity present prior to alkali treatment was tetraethylpyrophosphate, as proposed by Gazzard *et al.* [6], its hydrolysis product would be diethyl phosphate, which is not chloroform-extractable from neutral aqueous solution, and the values of Table 1 would represent the true dissociation constants.

The possibility that hydrophobic interactions play a significant role in the formation of the enzyme–inhibitor complex of other organophosphates cannot, of course, be ruled out on the basis of these findings. Agents with significant phosphorylating power might provide a more useful basis for studies of the effect of alkyl chain length.

The results presented here confirm the reports indicating that compounds of types I and II do not phosphorylate acetylcholinesterase and, since their action as reversible inhibitors is very weak, interesting questions arise concerning the reasons for their toxicity [6].

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Serum binding of methyltetrahydrofolic acid

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The binding of folates to human plasma is complex [1–4]. However, the exact percentage of bound and free folate in human plasma *in vivo* is unknown. It might be important to determine the per cent of unbound plasma folate as opposed to total plasma folate, because only unbound plasma folate could readily pass into the tissues [5]. An example of a drug where it is helpful to measure the unbound as opposed to the total plasma drug level is diphenylhydantoin [6]. Therapeutic results are better correlated with the unbound diphenylhydantoin in plasma than with the total plasma diphenylhydantoin level [6].

The purpose of this study was to measure the percentage of unbound folate in fresh human serum and plasma under various conditions. This was accomplished by utilizing a rapid, ultrafiltration method that controls pH and protects labile substances from oxidation [5, 7]. To simulate plasma folate levels after the oral ingestion of folates, radiolabeled methyltetrahydrofate (MeTHF) was added to fasting plasma or serum. It is known that ingested folates are rapidly converted in the body to MeTHF, the principal folate of human plasma [8].

^{14}C-(±)-L-*N*-5-MeTHF (60 mCi/m-mole) and ^{3}H-folic acid (16 Ci/m-mole) were obtained from Amersham/Searle. ^{3}H-(+)-L-*N*-5-MeTHF (170 mCi/m-mole) was extracted and purified from fresh rat liver the day after the injection of 0·2 mCi ^{3}H-folic acid [9, 10]. Nonradioactive (±)-L-*N*-5-MeTHF was obtained from Sigma Co.

The methods employed to ultrafilter serum or balanced salt solutions have been described previously [5, 7]. Briefly, 2·5-ml samples of a balanced salt solution or serum containing radiolabeled MeTHF were placed in a 2·5-ml chamber. With constant stirring and the application of a constant pressure head of 5 pounds per square inch (p.s.i.) using a 95% N_2 and 5% CO_2 gas mixture, the balanced salt solution or serum (termed the parent solution) was ultrafiltered through a Millipore filter (PSED-01310) at 23°. The first 200 μl of ultrafiltrate was discarded and the second 200-μl aliquot retained for analysis. Recovery of ^{3}H- or ^{14}C-MeTHF in the second 200-μl aliquot of ultrafiltrate in the absence of protein was determined by adding ^{3}H- or ^{14}C-MeTHF to balanced salt solution to yield final concentrations of 2 ng/ml, 25 ng/ml and 20 μg/ml. The balanced salt solution also contained 5·0 mg sodium ascorbate and 0·2 mg thiourea per ml to protect the MeTHF from oxidation. Duplicate 100-μl aliquots of the second 200 μl of ultrafiltrate as well as the parent solution in the chamber (before and after the ultrafiltration) were assayed for radioactivity [5, 7].

To determine serum binding, fresh serum was obtained from two healthy overnight-fasted male donors. Immediately after centrifugation, 2 ml serum was added to 0·5 ml of a balanced salt solution. After mixing, an appropriate amount of ^{14}C- or ^{3}H-MeTHF (1–10 μl) was added, and this solution (the parent solution) was ultrafiltered. Two 100-μl aliquots of parent solution (before and after the filtration) and ultrafiltrate were assayed for radioactivity. The fraction of free radiolabeled MeTHF was calculated after correction for quench and nonspecific binding by the filter (*vide infra*) by dividing the dis/min in the filtrate by the average dis/min in the parent solution taken before and after the filtration. In another series of experiments, no radioactivity was added to the fasting serum, and the folate activity in the parent solution and ultrafiltrate was assayed by a competitive protein binding assay using β-lactoglobulin of milk [11].

Experiments were also performed to measure the ^{14}C-MeTHF binding to fresh human serum by ultrafiltration with the following variations: the temperature was decreased to 4° or increased to 43°; or sodium diphenylhydantoin or sodium salicylate or sodium probenecid was added to the parent solution to achieve concentrations of 0·2, 3·0 or 1·0 mM respectively. In order to measure ^{14}C-MeTHF binding to solutions other than fresh human serum, another series of experiments was performed with the following solutions substituted for fresh human serum: frozen human serum (1 year old), fresh human plasma, fresh rabbit plasma, and recrystallized albumin (4 times) at a concentration of 4 g/100 ml of balanced salt solution, which also contained 0·1 mg/ml of thiourea and 0·2 mg/ml of sodium ascorbate to protect the MeTHF from oxidation.

In order to be certain that no conversion or deterioration of the ^{14}C- or ^{3}H-MeTHF occurred during the ultrafiltration, both isotopes were submitted to paper chromatography [Whatman No. 1 paper; 0·1 M potassium phosphate buffer, pH 6, with 0·5% (v/v) mercaptoethanol (M.E.)] and/or thin-layer cellulose chromatography in two systems: (1) 3% NH_4Cl (w/v), pH 6·2, with 0·5% M.E.; and (2) 0·1 M potassium phosphate buffer, pH 6, with 0·5% M.E. [12]. In all three systems, the racemic ^{14}C-MeTHF received from the company (and dissolved in 2 ml distilled H_2O with 4 mg sodium ascorbate, pH 6) appeared as a single peak corresponding to carrier MeTHF. In six samples of ultrafiltrate, the ^{14}C appeared as a single peak corresponding to carrier MeTHF. In the two thin-layer systems, the naturally occurring stereoisomer, (+)^{3}H-MeTHF, appeared as two peaks with greater than 90 per cent appearing on a large peak cor-