jection to the reduction of the intensity caused by this filtering.

A Cl^{36} sample, prepared as described, was compared with a radioiodine sample, using different counters which happened to be readily available in our institute. The results are seen in Table 1.

TABLE 1

Counter type	Window thickness mg/cm²	Ratio of cpm with standard and with radioiodine
Tracerlab	2.73 (mica)	1.08
Beta counter	1.5 (mica)	1.06
X ray counter	4.3 (mica)	1.06
Philips Eindhoven	about 80 (steel tube)	1.41

It is seen that the agreement with mica window counters is excellent, and even with a steel tube counter, a type of instrument which will never be chosen for standardizing radioiodine, the agreement is not worse than the average result obtained in different laboratories (*1*). This means that, with our standard, radioiodine samples can be counted under different counting arrangements without corrections.

The standard should not be used at a distance much less than half the diameter of the counter's sensitive surface. Results are best between this distance and twice its value; if larger variations are allowed results tend to be less satisfactory and deviations of about 25% may occur.

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The Inhibition of the Cholinesterase Activity of Human Blood Plasma by Neutral Phosphate Esters. II: Studies with Hexa 1-C^{14}-Ethyl Tetrapolyphosphate[1]

Ralph W. Brauer and Rita L. Pessotti

Department of Pharmacology and Experimental Therapeutics, School of Medicine, Louisiana State University, New Orleans

Compounds containing the grouping P-O-R (R = alkyl or aryl) are potent inhibitors of human plasma cholinesterase if they also include a sterically strained configuration around the phosphorus atom (as in tri *o*-cresyl phosphate), or if that atom participates in an acid anhydride linkage (as in the case of the tetraalykl pryophosphates) (*2*). The reaction between enzyme and inhibitor results in the rapid inactivation not only of the enzyme, but also of the inhibitor. Inactivation of the inhibitor is observed only on reaction with active esterase, and inactivation of the enzyme does not take place if the pyrophosphate linkages of compounds like tetraethyl pyrophosphate have been hydrolyzed by even brief contact of the inhibitor with water. The inhibition of plasma cholinesterase activity by such compounds is not reversed on removal of the inhibitor by hydrolysis and prolonged dialysis.

These observations strongly suggest that the interaction of plasma cholinesterase with such phosphorus compounds results in the formation of rather stable compounds involving the active groupings of the enzyme.

TABLE 1

Specific activity of HETP	7.02×10^2 cpm/mM
Activity added as HETP to all preparations	5.74×10^3 cpm/mg protein
Activity recovered in protein precipitated after dialysis:	
From active enzyme + intact HETP	8.05 cpm/mg protein
From active enzyme + hydrolyzed HETP	1.43 cpm/mg protein
From denatured enzyme + intact HETP	1.03 cpm/mg protein

In order to further test this hypothesis, hexaethyl tetrapolyphosphate (HETP) containing P^{32} or C^{14} as tracer atom has been prepared. An earlier test employing P^{32} tagged material (*2*) merely demonstrated that less than 1.0% of the HETP added was fixed on the enzyme, suggesting that the anticholinesterase activity of this material might reside in a minor component obtained in the course of preparation; possibly this component could be tetraethyl pyrophosphate (TEPP), the most active member of this group detected so far. The purpose of the present note is to report results obtained under more favorable conditions involving the use of C^{14}-labeled HETP.

The preparation of the inhibitor from P_2O_5 and $P(O)(OC_2H_5)_3$ was carried out as described previously (*2*), except that C^{14}-containing triethyl phosphate, prepared by reacting $CH_3C^{14}H_2I$ with silver phosphate, was employed.[2] The preparation obtained had a specific activity of 3 mc/mM of hexaethyl tetraphosphate. The human plasma esterase preparation employed[3] had a cholinesterase activity of 5.24 M CO_2 evolved/hr/g under standard conditions (0.8 M acetyl choline bromide) (*1*); this preparation is approximately ten times as pure as the fraction IV-6-3 previously employed.

The plan of the experiments, based upon these methods may be seen from Table 1. The following are additional details of preparation: a slight excess of inhibitor, or its equivalent, was added to the enzyme; HETP was hydrolyzed by contact with water (1% solution) for 72

[1] Supported by a grant from the Division of Research Grants and Fellowships, U. S. Public Health Service.

[2] This preparation was carried out for this laboratory by Tracerlab, Inc., Boston, Massachusetts.

[3] This material was made available through the kindness of Dr. Surgenor, Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts.

hr; less than 0.1% of the initial anticholinesterase activity remained at this time. Denatured enzyme was prepared by heating the enzyme solution to 80° C for 20 min to complete inactivation; 0.45% concentrations of protein were used in all cases. Dialyses were carried out in a rotating rack against 15 successive changes, at 8-hr intervals of 0.3% NaCl (30 ml per 4 ml enzyme solution each time); no C^{14} could be detected in any dialyzates after the tenth change. Precipitation of the protein from the dialyzed solutions was performed by the addition of five volumes of acetone to one of enzyme solution, after preliminary trial had shown that results obtained by this procedure did not differ from those obtained under the much more elaborate conditions required to obtain a dry precipitate of native protein. For counting, samples weighing 2.8–3.1 mg were collected on filter paper disks of 3.9 cm^2, dried in a vacuum desiccator, and counted directly (2.0 mg/cm^2 end window counter). Since all samples fell within the same narrow and low range of mass thickness, no self-absorption correction was applied. A standard was prepared by combustion of a known weight of HETP, collection of the CO_2 formed as $BaCO_3$, dilution of the activity with a known proportion of $BaCO_3$ in a homogenizer, and counting of a 3.0-mg sample on filter paper.

Three experiments carried out by these procedures yielded results in close agreement. Table 1 shows the averages of the values obtained. Three points are immediately evident from inspection of the figures. There is a definite, measurable uptake of C^{14} by the enzyme on reaction with active inhibitor. There is much less, though yet measurable, protein fixation of C^{14} under conditions which do not result in enzymologically detectable reactions between protein and phosphorus compounds. Only a small fraction of the HETP required to inhibit the enzyme is actually bound by the protein.

From the figures for C^{14} uptake and for the specific activity of the sample of HETP employed, molecular relations can be calculated if the molecular weight of the protein is known. Taking 3×10^5 as, at present, the most likely value for the mean molecular weight of this protein, a figure of 3.45×10^{-2} mol of HETP bound per mol of protein is obtained.

Assuming that inhibition of 1 mol of enzyme involves the firm fixation of 1 mol of inhibitor, these data suggest that the protein preparation employed actually contained about 3% active enzyme. This estimate is in good accord with values derived from other considerations.

The protein-to-inhibitor ratio derived from the fixation of C^{14} is also in good accord with the ratio calculated for TEPP from data on enzyme inhibition. In this case, 50% inhibition of the present enzyme preparation occurs at a ratio of 1.41×10^{-2}M TEPP/M protein, or a calculated uptake for complete inhibition (in view of the linear concentration-inhibition curve [*2*] of 2.82×10^{-2}M TEPP/M protein). This figure is in fair agreement with that calculated above if correction is made for C^{14} uptake that does not appear related to the enzyme-inhibitor reaction.

The data presented indicate the formation of a stable compound between plasma cholinesterase and a small fraction of the hexaethyl tetrapolyphosphate required to inhibit the enzyme. From 82 to 88% of the C^{14} fixation thus obtained appears to be related to the enzyme inhibition, the rest being accounted for by a much less specific reaction. Calculations have been presented indicating that the amount of C^{14} taken up in connection with the specific reaction is close to the amount of tetraethyl pyrophosphate required for complete inhibition of the enzyme, again suggesting that the anticholinesterase activity may reside in this or closely similar compounds of this group. Finally, an estimate of the molecular relations between HETP and the enzyme has been presented; a reasonable value (about 3%) for the purity of the enzyme preparation employed could be arrived at on the assumption of a mean molecular weight of 3×10^5 for the protein, and of a reaction involving 1 mol of HETP and 1 mol of protein.

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Iron Metabolism.[1] Heme Synthesis *in Vitro* by Immature Erythrocytes

R. J. Walsh, E. D. Thomas, S. K. Chow, R. G. Fluharty, and C. A. Finch

Department of Medicine, Harvard Medical School, The Medical Clinic, Peter Bent Brigham Hospital, Boston, and Radioactivity Center of the Laboratory of Nuclear Science and Engineering, Massachusetts Institute of Technology, Cambridge

Studies with radioiron have indicated that there is no exchange of iron between the mature erythrocyte and surrounding plasma (*3, 4*). The following data, however, indicate that reticulocytes will assimilate iron and synthesize heme *in vitro*, and that this uptake of radioiron may be used as an indicator of the rate of hemoglobin synthesis.

Fe^{59} and Fe^{55} with specific activity of about 20 and 200 μc/mg iron respectively were prepared at the Massachusetts Institute of Technology cyclotron. Blood with an increased content of reticulocytes from patients with pernicious anemia during a response to liver and from patients with iron deficiency, and bone marrow from rats and humans were used. *In vitro* studies were performed in rocking boats at 37° C in a gas mixture of 95% oxygen and 5% CO_2, as previously described by Geiman and his associates (*2*), over a period from 4 to 24 hr. Those experiments extending beyond 6 hr were carried out with sterile precautions.

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