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SPECIFIC RADIOISOTOPIC ASSAY FOR CHOLINESTERASE

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May 1990

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The radiometric method (I) for measuring ChE activity [Siakotos et al., Biochem. Med. 3, 1, 1969] was modified to preclude the use of p-dioxane, a hazardous material. The modified procedure (II) uses 0.4 N perchloric acid (PCA), instead of p-dioxane, to denature the ChE and stop hydrolysis of 14C-acetylcholine (ACh). The unreacted substrate (ACh) is removed by cationic exchange resin suspended in water. The supernatant (acidic water solution) containing the product of hydrolysis, ^{14}C-acetic acid, is mixed with nonhazardous scintillation cocktail and counted. The incubation mixture (37 degrees C) for II is similar to I and is composed of 0.1 ml of buffer-salt solution (pH 7.8), 0.1 ml of guinea pig whole blood (WB)-water suspension and 0.1 ml of 3mM ACh solution. Procedures I and II were compared to a titragraphic assay for ChE activity; specific activity values of WB (umoles ACh hydrolyzed/ml/hr) were found to be 72.4, 137.6 and 135.0, respectively. When ^{14}C-acetic acid was processed through procedures I and II, significantly less ($p<0.05$) ^{14}C was found in the supernatant from I, whereas all of the expected ^{14}C was found in the

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supernatant of II, suggesting that resin in dioxane will remove significant amounts of the product of hydrolysis; this finding may explain the observed difference in specific ChE activity between I and II. In summary, our modified procedure II is sensitive, precise and utilizes aqueous PCA instead of p-dioxane.

FOREWORD

This research was done under protocol 1-02-86-004-A-389, entitled "Measurement of Inhibitory Potency of Novel Anticholinesterase Compounds using Whole Blood or Erythrocyte Acetylcholinesterase from Several Species," under Project No. 3M263002D, Task Area No. 995BA. We acknowledge the participation of SPC Glen Jackson and Karl Ford in this project.

OBJECTIVES AND LOCATION OF DATA

Objective: The objective of these experiments was to establish a sensitive and precise method for measuring cholinesterase activity that would preclude the use of the hazardous solvent p-dioxane.

Location of data: The data are located in notebook 019-88 and in file folders held by L.W. Harris, D.R. Anderson and W.J. Lennox.

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INTRODUCTION

Since the early 1960s, several radioisotopic assays have been described for determination of cholinesterase (ChE) activity.[1-6] In 1968, McCaman et al.[6] described a sensitive radiometric procedure capable of measuring ChE activity in microliter or milligram samples of tissue; in the procedure ^{14}C-acetic acid from the hydrolysis of the substrate, acetyl-1-^{14}C-choline (ACh), is measured and the unhydrolyzed substrate is quantitatively removed as an insoluble Reinecke salt. In two of the earlier methods,[3,4] ion exchange resin in ethanol was used to remove the unreacted substrate.

In 1969, Siakotos et al.[7] reported further improvement of the radiometric procedure. In this improvement, unreacted substrate, ACh, is removed on Amberlite CG-120 resin suspended in p-dioxane, and according to the authors, the blanks are lower and the method is more precise than earlier methods. As in the earlier methods, the hydrolysis product (free, ^{14}C-labeled acetic acid in the supernatant solution) is measured in a liquid scintillation counting system.

The method of Siakotos et al.[7] has been used extensively by our institute[8-10] and by scientists elsewhere.[11,12] We have found the procedure to be simple, precise in estimating relative ChE inhibition and reproducible among assayists. However, the use of p-dioxane in the method poses some problems in storage, handling and disposal. Foremost, p-dioxane is considered a carcinogen and a hazardous material by the EPA.[13,14] As a result, procedures for disposal of radioactive waste materials contaminated with p-dioxane are becoming complex and expensive. For these reasons, we have recently developed a radiometric ChE assay using water as solvent and a nontoxic, nonhazardous, nonflammable and biodegradable cocktail for scintillation counting. The purpose of the study reported herein was to compare our radiometric ChE assay to the radiometric method of Siakotos et al.[17] using Amberlite IRP-69 resin, and each of these radiometric methods to the titragraphic method[15] (TG).

MATERIALS AND METHODS

Materials. Physostigmine salicylate (Phy) was supplied by the Walter Reed Army Institute of Research, Washington, DC. The anticholinesterase S-2-N,N-Diethyl-N-methylammonioethyl-O-pinacolyl methylphosphonothioate methylsulfate (DMPMM) was synthesized under contract by Ash-Stevens Inc.[16] IRP-69 resin was purchased from Rohm & Haas, Philadelphia, PA; chemically it is identical to CG-120 resin, but the mean particle size is larger. The scintillation cocktail (Ecolume®) was purchased from ICN Radiochemicals, Irvine, CA. The substrate acetyl-1-^{14}C-choline iodide (2.4 mCi/mmole) was obtained from New England

Nuclear Corp., Boston, MA, while p-dioxane was obtained from J.T. Baker Chemical Co., Phillipsburg, NJ. Perchloric acid (70% w/w) was purchased from EM Science, Cherry Hill, NJ. Anticoagulant-treated guinea pig whole blood, erythrocytes therefrom, and eel AChE were used as the sources of ChE. Blood was obtained from guinea pigs under protocol 1-02-86-004-A-389; the blood was drawn via cardiac puncture, under ether anesthesia, as per DF, dated 27 April 1986, to chairman, Laboratory Animal Care and Use Committee. The service was performed by veterinarians of the Veterinary Medicine and Laboratory Resources Division. The eel AChE in solution (1000 units/mg protein) was obtained from Sigma Chemical Co., St Louis, MO; Hartley strain guinea pigs of both sexes were obtained from Charles River Laboratories, Kingston, NY.

Methods. In preparation for the assays, a slurry of the IRP-69 resin was prepared by mixing 20 g with sufficient water or dioxane to make 100 ml. The ^{14}C-ACh was dissolved in 1 mM acetate buffer at pH 4.5; sufficient unlabeled ACh chloride was added to give a total ACh concentration of 3 mM and an activity of 1 μCi/ml of solution. The stock solution of ACh was subdivided into 20 ml aliquots and stored at -20°C. A sample was thawed and stored on ice for use during an enzyme assay; the ACh was returned to the freezer at the end of the assay. Each sampling of enzyme activity was performed in duplicate, and the mean of the pair of values was calculated; the n value in each table and figure herein indicates the number of such duplicate samplings performed.

The two radiometric methods and the TG method were run in parallel using guinea pig whole blood to compare specific ChE activities. Also, two inhibitors of ChE were used to test the capability of each radiometric procedure to quantitate ChE inhibition; the inhibitors were the carbamate Phy and DMPMM, an organophosphorus anticholinesterase. Fresh stock solutions of DMPMM (0, 0.1, 0.25, 0.5, 1, 1.5 and 2 x 10^{-4}M) or Phy (0, 0.025, 0.05, 0.1, 0.25, 0.5 and 2 x 10^{-4}M) were prepared daily in saline and stored on ice; ten μl of each concentration of inhibitor solution was added to 1 ml portions of whole blood, mixed, and incubated for 30 min at 37°C. The samples were then removed from the incubator and placed on ice. Immediately thereafter, four aliquots (25 μl each) of inhibited blood were removed from each sample and placed into tubes, each containing 75 μl of twice distilled water, 100 μl of 0.1 M PO_4 buffer (pH 7.8, containing 0.3 M NaCl) and 100 μl of ^{14}C-ACh stock solution, for ChE assay by the two radiometric methods. Following addition of the labeled ACh, each tube was incubated for either 5 (perchloric acid procedure) or 7 (dioxane procedure) min at 37°C; these incubation times yielded equal hydrolysate activity, approximately 10,000 dpm. Hydrolysis of substrate was then stopped by the addition of either 100 μl of 0.4 N perchloric acid (PCA) followed by sufficient water/IRP-69 resin to make 5 ml or

dioxane/IRP-69 resin to make 5 ml. Tubes were then brought to 10 ml with additional water or dioxane and centrifuged; two ml (PCA method) or 5 ml (dioxane method) was transferred into 10 ml of scintillation fluor for counting. Parallel blanks (no enzyme) were run to correct for spontaneous hydrolysis of substrate during the incubation period.

In preparing erythrocytes for enzyme assay, inhibited whole blood samples were each washed with 10 ml of cold saline, followed by centrifugation for 5 min at 2,300 x g (3,000 rpm in an IEC, DPR-6000 centrifuge). After removing the supernatant, 15 µl aliquots of sedimented erythrocytes were removed, combined with 85 µl of twice distilled water, and assayed for ChE activity using the two radiometric methods described above.

Preliminary comparisons of the two methods revealed that higher dpm values were found in supernatant solutions from samples treated with PCA, suggesting that PCA might be hydrolyzing the ACh. This possibility was tested, at room temperature, by preparing 20 tubes, each with 0.3 ml of buffer/water/^{14}C-ACh stock substrate (100 µl of each component). At 20-sec intervals, 100 µl of 0.4 N PCA (0.1 N final concentration) or water was added. Immediately after treating the last sample, 5 ml of water/resin was added to each of the first four samples, and they were further processed for counting as described above. The process was repeated on additional sets of four, at 5-min intervals, until all 20 samples were processed.

Another possible reason for higher counts in PCA-treated samples is that the functional moieties of the resin might be interacting differently with the ^{14}C-labeled ions depending upon the suspending medium, dioxane or water. To investigate this hypothesis, two 6-ml preparations of water/^{14}C-ACh were made. The first preparation contained 4 ml of water and 2 ml of the ^{14}C-ACh stock solution; it was incubated for 30 min at 37°C. Each of five 100 µl aliquots was transferred to a scintillation vial containing 10 ml of fluor for measurement of total dpm. Ten 0.3 ml aliquots were placed into tubes; five ml of dioxane/resin slurry was added to each of 5 tubes, while 100 µl of 0.4 N PCA solution followed by enough water/resin slurry to make 5 ml was added to each of the other 5 tubes. Each of the 10 tubes was brought to 10 ml with the appropriate vehicle, dioxane or water, mixed and centrifuged as before; two ml (each PCA tube) or 5 ml (each dioxane tube) of supernatant solution was put into 10 ml of fluor and counted.

The second 6-ml preparation was made by adding 50 µl of eel AChE solution to the tube, adding sufficient water to make 4 ml and then adding 2 ml of ^{14}C-ACh stock solution; this tube was also incubated for 30 min at 37°C. (The eel AChE hydrolyzes virtually all of the ACh.) The contents were then subdivided and further treated as stated above for the first preparation.

The specific activity of ChE can be accurately measured by the titragraphic method;[15] the automated titragraphic equipment measures and adds the amount of KOH (0.01 M) solution required to maintain a constant pH during the course of the reaction. This method was used as the standard for comparison of ChE activities. Briefly, the system was calibrated at 37°C using National Bureau of Standards buffers of pH 4.00 and pH 7.00. The set point was maintained at pH 7.8 by the addition of 0.01 M KOH solution. The normality of the base was determined by titration of 100 µl of 0.01 M potassium phthalate solution. The substrate was acetylcholine Cl (1 mM) in saline. The substrate solution was admitted to the reaction vessel, and 45 µl of 0.01 M KOH solution was added to bring the substrate closer to the set pH of 7.8, followed immediately by the addition of 20 µl of guinea pig whole blood. The rate of hydrolysis was measured for 7 min, but the first 4 min were used to stabilize the instrument at the set pH; only the last 3 min of the hyrolysis curve, which was linear, were used to determine the specific AChE activity.

A repeated measures analysis of variance[17] was used to test for significant differences between the two radiometric methods, between the two tissues and among the six concentrations of inhibitor, as well as for the interactions among these factors. A one-way analysis of variance followed by Newman-Keuls testing of the means was used to evaluate differences among all three methods used to measure ChE activity.

RESULTS

Figure 1 illustrates the effects of various concentrations of Phy on guinea pig whole blood and erythrocyte ChE activity using the water/resin and dioxane/resin systems for the removal of unhydrolyzed ACh; Figure 2 also represents a similar study in which DMPMM, also an irreversible, but reactivatable inhibitor of ChE, was used. Analysis of the data revealed no significant difference between the two methods for either inhibitor. The data in Figure 2 also reveal that DMPMM inhibited erythrocyte ChE more than whole blood ChE.

The data in Table 1 demonstrate that, when compared with water, 0.1 N PCA does not significantly hydrolyze ACh during the 20-min test period. This test period was chosen because it is expected that in future enzyme assays all samples will receive resin no later than 20 min after addition of PCA.

Table 2 shows that both the water/resin and dioxane/resin systems were quite efficient in binding unhydrolyzed ^{14}C-ACh. [Assuming the same total dpm with and without resin, percent bound = (dpm without resin - dpm with resin) ÷ (dpm without resin) x 100.] There was only a slight difference, 3.4, in percent of added radioactivity bound by resin. However, under

the reasonable assumption that the radioactivity of unbound material in supernatant solution is that of ^{14}C-acetic acid from the spontaneous hydrolysis of labeled ACh, such radioactivity from the dioxane/resin system was only 5,958 + 14,400 = 0.41 times that from the PCA-water resin system. The data in Table 3 may provide an explanation; these data reveal that the acid followed by water/resin bound virtually no ^{14}C, while significant ($p<0.05$) binding occurred with dioxane/resin. The ratio of dpm for dioxane/resin to dpm for acid followed by water/resin in Table 3 is 126,555 + 214,296 = 0.59, which is roughly similar to that in Table 2, or 0.5 overall.

A comparison of the TG assay with the two radiometric methods for determination of specific ChE activity of guinea pig whole blood is presented in Table 4. The specific ChE activity by the acid radiometric procedure was virtually identical to that by the TG method, whereas the activity using the dioxane/resin method was significantly lower ($p<0.05$).

DISCUSSION

The radiometric procedure for determination of ChE activity reported here differs from that of Siakotos *et al.*[7] in two respects. First, the reaction is stopped by using perchloric acid before the resin to denature the ChE instead of by coincident denaturation of ChE by p-dioxane and adsorption of unreacted substate by rsin. Second, water instead of p-dioxane is used as the vehicle for suspension of the resin. These steps have completely eliminated p-dioxane, a hazardous material and potential carcinogen, from the assay and thereby resolved the disposal problem.

When the two methods were run in parallel to estimate ChE inhibition caused by various concentrations of Phy or DMPMM, no significant differences ($p>0.05$) in relative inhibition were observed (Figures 1 & 2). However, it was found that dioxane/resin removed a significant amount of the product of hydrolysis, ^{14}C-labeled acetic acid, from the supernatant (Table 3). Because the ratios of the dpm values (resin in dioxane/resin in water) with slight (Table 2) and essentially complete hydrolysis of the ^{14}C-labeled substrate (Table 3) were roughly similar, the relative ChE inhibition values determined by the two methods were similar, as expected, using Phy or DMPMM. However, nonspecific removal of the acetic acid by dioxane/resin presents problems when comparing specific activities by the different methods (Table 4). Note that the specific ChE activity using dioxane/resin is only 53% of that obtained with acid followed by water/resin or with the standard TG method.

In summary, a different radiometric method for measuring ChE activity is presented. The method utilizes perchloric acid to

stop the enzymatic reaction and a water slurry of IRP-69, cationic exchange resin to remove the unreacted substrate (^{14}C-ACh). The aqueous supernatant containing the product of hydrolysis (free, ^{14}C-labeled acetic acid) is mixed with a nontoxic, nonflammable, biodegradable scintillation cocktail for counting. The method is recommended for routine assays to obviate the use of dioxane.

Table 1

Effect of 0.1 N Perchloric Acid on Hydrolysis of ^{14}C Acetylcholine

Contact Time (min)	Condition in Aqueous Solution*			
	Acid Present		Acid Absent	
	Mean DPM	δ	Mean DPM	δ
5	2633	650	2853	78
10	2913	64	2981	254
15	2977	17	2940	335
20	3033	18	3078	499
25	2995	20	2872	192

* Room temperature, 25°C; 0.75 mM (final) ^{14}C-ACh (0.25 μCi/ml); for each condition and contact time, a pair of samples was removed from the initial preparation and counted.
δ = within pair difference in DPM.

Table 2

Comparative Binding of ^{14}C Acetylcholine by IRP-69 Cationic Exchange Resin

Method (n=5)	DPM (mean $\pm$ s.d.)	Percent Bound
Perchloric Acid-Water		
With Resin*	14400 $\pm$ 542	94.1
Without Resin	242397 $\pm$ 30939	
Dioxane		
With Resin	5958 $\pm$ 145	97.5
Without Resin	242327 $\pm$ 16908	

* n=4

Table 3

Comparative Binding of ^{14}C-Acetic Acid (^{14}C-Acetylcholine* Hydrolysate) by IRP-69 Cationic Exchange Resin

Suspending Medium	DPM Mean ± s.d. (n=5)
None (No Resin)	215660 ± 4992
Perchloric Acid-Water	214496 ± 4981
Dioxane	126555 ± 1769

* The ACh was completely hydrolyzed by eel acetylcholinesterase (200 µl eel AChE solution, 15 mmoles/ml/hr) before measurement of the radiolabel.

Table 4

Comparison of Specific Activity Estimates for Guinea Pig Whole Blood Acetylcholinesterase Obtained by Three Assay Methods

Method	Specific Activity mmoles of ACh hydrolyzed/ml WB/hr mean ± s.d. (n=12)
Radiometric (pH 7.8)	
Resin in Dioxane	72.4* ± 6.1
Resin in Water	137.6 ± 12.0
Titragraphic (pH 7.8)	135.0 ± 12.8

* Differs significantly, $p < 0.005$, from the means of the other two methods.

FIG. 1. Comparative Effects of IRP–69 Resin in Water and in Dioxane on Radiometric Assay Estimates of Cholinesterase Activity of Guinea Pig Blood Inhibited by Physostigmine Salicylate

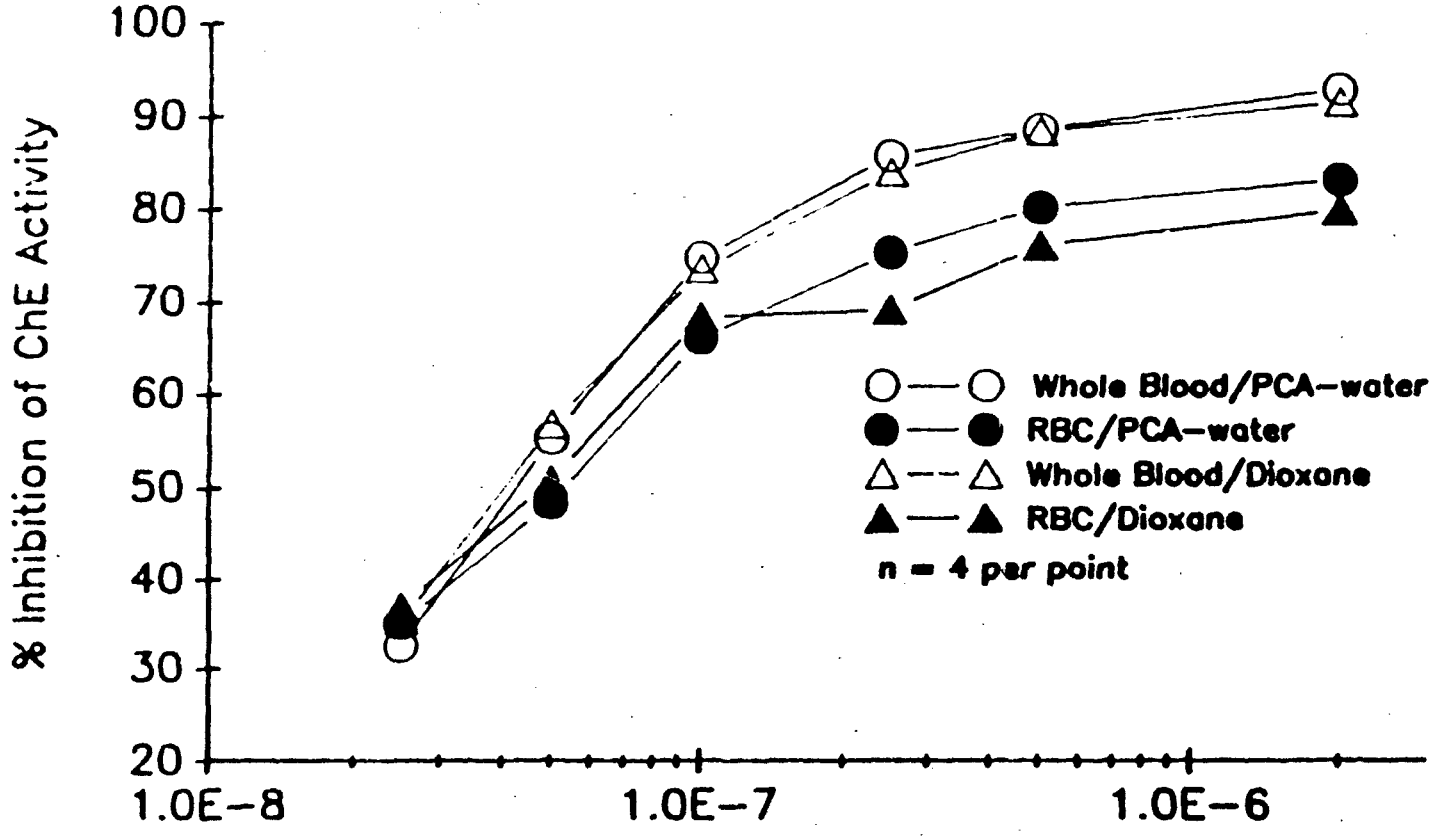
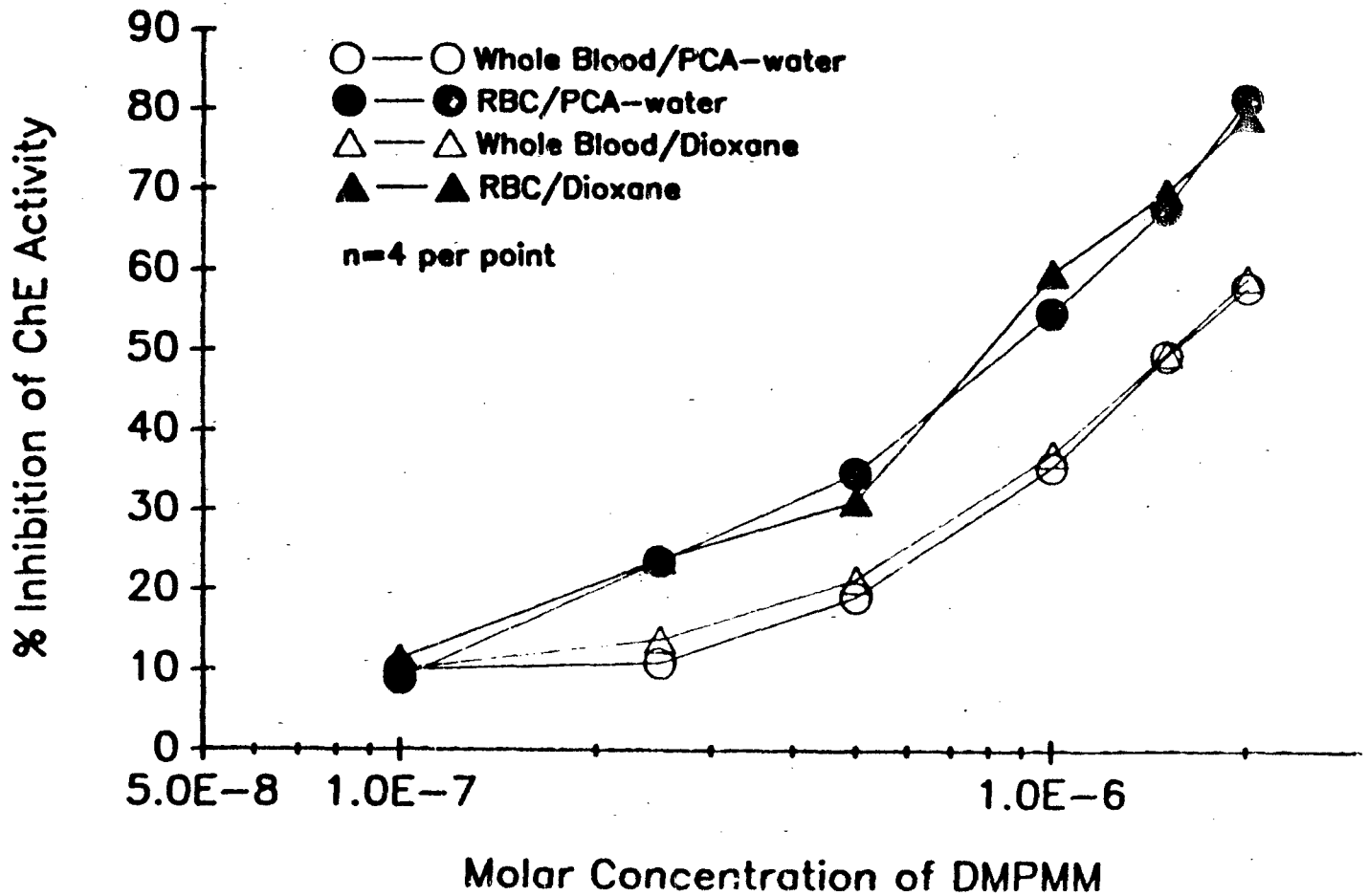


FIG. 2. Comparative Effects of IRP–69 Resin in Water and in Dioxane on Radiometric Assay Estimates of Cholinesterase Activity of Guinea Pig Blood Inhibited by S–2–N,N–Diethyl–N–Methylammonioethyl–O–Pinacolyl Methylphosphonothioate Methylsulfate (DMPMM)



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