protein is limiting only with respect to threonine and total sulfur.

SUMMARY

A. priceana tubers are unique in their large size and ability to grow in highly alkaline, wooded habitats. To obtain a protein that can be useful in human nutrition, the nonprotein nitrogen of the tubers can be extracted by alcohol. *A. priceana* would appear to be a better germplasm source for breeding with other *Apios* species to expand their habitats.

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Effect of Chain Length and Concentration on the Degree of Dissociation of Phosphates Used in Food Products

Graham R. Trout[1] and Glenn R. Schmidt*

A sodium ion selective electrode was used to determine the degree of dissociation of six different sodium phosphates commonly used in food products. The phosphates investigated had number average chain lengths between 1.0 and 20.8. All phosphates were analyzed at pH 6.0 and at four concentrations between 0.15 and 0.60%. The results showed that the concentration had no significant ($p > 0.05$) effect on the degree of dissociation of the phosphates, whereas the chain length had a large significant effect ($p < 0.001$). The degree of dissociation decreased as the chain length of the phosphate increased; the rate of decrease was proportional to the square of the chain length. The values for the degree of dissociation ranged from 91.6% for the shortest chain length phosphate to 38.0% for the longest.

INTRODUCTION

Phosphates are used in processed meat products to reduce the amount of water lost during cooking and to improve the texture of the product (Ellinger, 1972). Although phosphates are very effective at increasing both of these functional properties, it is not fully understood how they achieve this. As a result, it has been difficult to determine which type of phosphate and what conditions are required for the phosphates to produce the maximum increase in either of these functional properties.

Phosphates increase the functional properties of meat products by one or more of the following ways: (a) by increasing the pH of the product; (b) by increasing the ionic strength of the product; (c) by dissociating actomyosin, the main structural protein of muscle, into actin and myosin; (d) by binding to the meat proteins (Hamm, 1970). But it is not known how important each of these factors are because the exact ionic strength of the phosphates cannot be calculated. This is because the ionic strength of the phosphates is determined by their degree of dissociation, which in turn is determined by the type and concentration of phosphate (Van Wazer and Callis, 1958). And to date, the values obtained for the degree of dissociation of the phosphates commonly used in food products have been quite variable (Wall and Doremus, 1954; Schindewolf, 1953; Schindewolf and Bonhoeffer, 1953; Batra, 1965). The values for the degree of dissociation of the phosphates must be known accurately because a small error in the degree of dissociation produces a large error in the value calculated for the ionic strength.

The sodium ion electrode is the most useful instrument available for measuring the degree of dissociation of phosphates at the concentrations typically found in meat products (0.1%-0.6% in the aqueous phase) (Gardner and Nancollas, 1969). In several previous studies, however, these electrodes have been used as concentration probes (Rechnitz and Brauner, 1964; Batra, 1965) rather than activity probes. When they are used as concentration probes, they produce erroneous results because at high phosphate concentrations the activity of the sodium ion

Department of Animal Sciences, Colorado State University, Fort Collins, Colorado 80523.
[1]Present address: Department of Animal and Dairy Sciences, Auburn University, Auburn, AL 36849.

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Table I. Common Name, Chain Length, Weight and Molar Concentration, Degree of Dissociation, and Pertinent Data Generated in Calculating the Degree of Dissociation of the Phosphates Studied

phosphate type	chain[a] length ($\bar{n}$)	[Phos],[b] % (w/v)	10^3[Phos],[c] M	10^4[HCl],[d] mol	10^2I	f_{Na^+}	10^3[Na^+],[e] M	dissoc,[f] %
disodium phosphate	1.0	0.15	10.53	9.17	1.779	0.8766	19.11	90.7
		0.30	21.06	18.34	3.608	0.8400	38.58	91.6
		0.45	31.59	27.51	5.453	0.8165	58.16	92.0
		0.60	42.12	36.68	7.254	0.8000	77.42	91.9
tetrasodium pyrophosphate	2.0	0.15	5.63	8.86	2.498	0.8597	20.09	89.3
		0.30	11.25	17.71	4.842	0.8234	39.56	87.9
		0.45	16.88	26.57	7.343	0.7993	59.67	88.4
		0.60	22.50	35.43	9.735	0.7831	79.33	88.1
sodium tripolyphosphate	3.0	0.15	4.04	5.32	2.658	0.8565	16.68	82.6
		0.30	8.08	10.65	5.136	0.8200	32.82	81.3
		0.45	12.12	15.97	7.697	0.7966	49.21	81.2
		0.60	16.16	21.29	10.201	0.7805	65.44	81.0
sodium tetrapolyphosphate	5.7	0.15	2.32	2.65	2.328	0.8634	11.38	63.5
		0.30	4.65	5.29	4.658	0.8256	22.79	63.6
		0.45	6.97	7.94	6.994	0.8021	34.19	63.6
		0.60	9.30	10.59	9.115	0.7869	45.09	62.9
sodium hexametaphosphate	12.8	0.15	1.10	0.92	2.248	0.8652	7.27	44.9
		0.30	2.20	1.83	4.789	0.8240	15.01	46.3
		0.45	3.30	2.75	7.355	0.7992	22.78	46.8
		0.60	4.39	3.67	8.995	0.7877	29.11	44.9
sodium phosphate, glassy	20.8	0.15	0.69	0.11	2.819	0.8535	5.97	38.2
		0.30	1.37	0.22	5.636	0.8147	11.95	38.2
		0.45	2.06	0.32	8.334	0.7921	17.78	37.9
		0.60	2.74	0.43	10.957	0.7765	23.56	37.6

[a] Phosphate number-average chain length. [b] Weight concentration of phosphate. [c] Molar concentration of phosphate. [d] Moles of HCl to titrate each phosphate to pH 6.00. [e] Concentration of sodium ions free in solution. [f] Degree of dissociation of the phosphates; percentage of the sodium free in solution.

is considerably less than its concentration. Depending on the ionic strength of the solution, the activity of the sodium ion can be up to 30% lower than its concentration and hence the calculated degree of dissociation is also lower by a similar amount. But when ion-selective electrodes are used as activity probes, and used accurately with careful standardization and rigorous experimental technique, they produce accurate and reliable results. For example, the dissociation constants of sodium trimetaphosphate and tetrametaphosphate determined at relatively high concentrations using the sodium ion electrode (Gardner and Nancollas, 1969) agree well with values obtained at much lower concentrations using a transference and conductance technique (Monk, 1949).

Therefore, the objective of this study was to use the sodium ion selective electrode as an activity probe to determine the degree of dissociation of six different phosphates with chain lengths between 1.0 and 20.8 at four different weight concentrations between 0.15% and 0.60%.

EXPERIMENTAL SECTION

Materials. The phosphates used in the experiment were all food grade (supplied by FMC Corp., Philadelphia, PA) with the exception of the disodium phosphate, which was AR grade. The number-average chain length ($\bar{n}$) of the food-grade phosphates was determined by the titration method outlined by Lowenheim (1973). In brief, this method involves (1) determining the number of terminal or end-group phosphate units by titrating the acidified phosphates between the end points at pH 4.5 and 9.0 and (2) determining the total number of phosphates units by hydrolyzing the phosphates in 6 N HCl and titrating them between the same two end points. From these titration data n was calculated by the following formula:

$$\bar{n} = 2(\text{total phosphate units})/(\text{end-gp phosphate units})$$

The number-average chain length, common name, weight concentration, and molar concentration of the phosphates investigated in this study are given in Table I. Information provided by the phosphate suppliers indicates that the pyrophosphate and tripolyphosphate used in this study are essentially homogeneous compounds, whereas the longer chain length phosphates are approximately normally distributed mixtures of phosphates of different chain length (for further details see: Westman and Gartaganis, 1957; Van Wazer, 1958). All other reagents used were AR grade. Solutions were prepared with deionized distilled water; carbon dioxide was excluded by bubbling with nitrogen gas.

Procedure. The sodium activity was measured with a sodium ion selective electrode (Fisher, Catalog No. 13-639-20) used in conjunction with a calomel electrode fitted with a porous plug (Fisher, Catalog No. 13-639-51). Diffusion of potassium chloride from the reference electrode was less then 3×10^{-5} mol of K^+/h and hence was not sufficient to produce a measurable change in emf (electromotive force) during measurement. Before use, the sodium ion electrode was aged in 0.1 M sodium chloride for 48 h. When not in use, the sodium ion electrode was stored in 0.1 M NaCl and the calomel electrode in pH 7.00 buffer. Measurements were made at room temperature (25 ± 1 °C) with a liquid junction as follows:

sodium ion electr/soln under study/calomel electr

emf values were measured with an Orion 901 Microprocessor Ionanlyzer and were reproducible to ±0.1 mV. The electrode system was standardized before use and after every hour of use, with several concentrations of sodium chloride covering the concentration range of interest. All standards were prepared in pH 6.0 NH_4Cl/NH_3 buffer so that the total ionic strength of the solutions was 0.12. Before analysis, the phosphates were titrated to pH 6.00 ± 0.01 with either 0.2 or 0.02 M HCl. This pH was chosen because it is the average pH of meat products that contain phosphates and also because it is considerably larger than the pNa and hence should not produce any hydrogen ion interference (Averill, 1983).

The experiment was designed to determine the effect of phosphate chain length (1.0, 2.0, 3.0, 5.8, 12.7, 20.8) and weight concentration (0.15%, 0.30%, 0.45%, 0.60%) on the

degree of dissociation of sodium phosphates. The treatments were arranged as a six by four complete factorial (six phosphate chain lengths by four concentrations), and hence 24 treatments combinations were investigated. The experimental design used was a randomized complete block design, with two blocks (replicates). Duplicate readings were made on each sample in each replicate. The calculated degree of dissociation values was analyzed by analysis of variance. When F values were significant, Fischer's least significant difference (LSD) method was used to locate differences between treatment means (Snedecor and Cochran, 1976). A polynomial was fitted to the data by using the method described by Snedecor and Cochran (1976).

Calculations. The degree of dissociation was calculated from the concentration of free sodium and total sodium:

$$\alpha = [Na^+]/[(\bar{n} + 2)[Phos]] \quad (1)$$

Activity coefficients were calculated by using the Davies (1962) extended form of the Debye–Huckel equation:

$$-\log f_{Na^+} = 0.509\left[\frac{I^{0.5}}{1 + I^{0.5}} - 0.3I\right] \quad (2)$$

From this, the sodium ion concentration was calculated:

$$[Na^+] = a_{Na^+}/f_{Na^+} \quad (3)$$

Ionic strength was calculated by

$$I = 0.5[[Phos_1](Z)^2 + [Na^+_1] + [H^+] + [Cl^-]] \quad (4)$$

Z, the charge on the phosphate molecule, was calculated as follows:

$$Z = [[Na^+]/[Phos]] - (H^+/Phos) \quad (5)$$

For computational purposes eq 1 and 3–5 were reduced to

$$I = 0.5[[Phos_1][[a_{Na^+}/[f_{Na} + [Phos]]] - (H^+/Phos)]^2 + (a_{Na^+}/f_{Na^+}) + [H^+] + [Cl^-]] \quad (6)$$

where α = degree of dissociation, $[Na^+]$ = molar concentration of free sodium, $[Na^+_1]$ = molal concentration of free sodium, [Phos] = molar phosphate concentration, $[Phos_1]$ = molal phosphate concentration, Phos = number of moles of phosphate, H^+ = number of moles of HCl to titrate the phosphates to pH 6.0, $\bar{n}$ = number-average phosphate chain length, f_{Na^+} = Na^+ molar or molal activity coefficient, a_{Na^+} = Na^+ activity, I = ionic strength. The sodium ion activity coefficient was calculated by successive approximations to eq 2 and 6; the calculations were started with an ionic strength value of 0.3 in eq 2 and continued until two consecutive computed values of f_{Na^+} differed by less than 1×10^{-5}. Once the sodium ion activity coefficient was obtained, the free sodium concentration was calculated by eq 3 and from this the degree of dissociation was calculated from eq 1; with the disodium phosphate, the $(\bar{n} + 2)$ term in eq 1 was replaced with $(\bar{n} + 1)$.

RESULTS AND DISCUSSION

Effect of Phosphate Weight Concentration. In the analysis of variance of the data there was no significant effect of phosphate concentration, either as a main effect ($p > 0.05$) or as an interaction ($p > 0.05$). Hence, in the concentration range studied (Table I) the phosphate concentration has no effect on the degree of dissociation of any of the phosphates. This conclusion is consistent with an earlier finding (Gardner and Nancollas, 1969) that the degree of dissociation of trimetaphosphate and tetrametaphosphate increases only slightly (1.5% and 4.5%, respectively) with a similar reduction (fivefold) in concentration. In our study, the reduction in concentration may have produced an increase in degree of dissociation of similar magnitude to that reported in the earlier study, but the increase was not detected due to variation in the data. The LSD ($p < 0.05$) between different concentrations of the same phosphate was 2.25%; hence, an increase in the degree of dissociation smaller than this would not be detected. An examination of the results in Table I, however, shows that there was no significant and consistent change in the degree of dissociation with concentration, with any of the phosphates. The change in concentration had no effect on the degree of dissociation of the phosphates most probably because the phosphates from sodium–phosphate ion pairs that are stable at very low concentrations ($<2.5 \times 10^{-4}$ M) (Gardner and Nancollas, 1969).

The results of two studies, one by Wall and Doremus (1954) and the other by Batra (1965), indicate that concentration has a greater effect on the degree of dissociation of phosphates than reported in this study. However, these effects were due to erroneous assumptions made by the researchers.

Wall and Doremus (1954) used transference and conductivity measurements to determine the degree of dissociation of different phosphates. In their calculations they assumed that teh molar ion conductivity of sodium (I_{Na^+}) varied with the square root of the sodium concentration. However, this is true only when the sodium concentration is less than 2×10^{-2} M (Daniels and Alberty, 1975) or possibly as high as 3.6×10^{-1} M, (Moore, 1975) but not in the concentration range they investigated (3×10^{-2} to 5×10^{-1} M). Compounding this error is the fact that these workers used a technique that is not suitable for determining ion association constants of salts at concentrations greater than 1×10^{-2} M (Johnson and Pytkowicz, 1979). Therefore, it is not surprising that the degree of dissociation values obtained by Wall and Doremus changed erratically with changes in phosphate concentration, particularly at the higher concentrations.

In a later study, Batra (1965) used the sodium ion electrode as a concentration probe to determine the degree of dissociation of different phosphates. However, when the sodium ion electrode is used in this manner, the values obtained for the degree of dissociation are much lower than those obtained in other studies and also the values change, misleadingly, as the phosphate concentration changes.

These erroneous results are obtained because the sodium ion electrode measures sodium ion activity not sodium ion concentration. The activity of the sodium ion is determined by the ion strength of the solution and decreases as the ionic strength increases (see eq 2 and 3). Because phosphates are polyvalent, they increase the ionic strength of the solution considerably, which reduces the activity of the sodium ion. For example, the activity of the sodium ion in a 0.02 M pyrophosphate solution is 30% lower than the molar concentration of the sodium ion. Because the sodium ion activity is lower than the sodium ion concentration (except at extremely low concentration), the degree of dissociation values calculated by using sodium ion activity instead of the sodium ion concentration will always be lower than the actual values (see eq 1). Also, with this method, the calculated values for the degree of dissociation increase as the phosphate concentration decreases, because the decrease reduces the ionic strength of the solution, which in turn increases the sodium ion activity. This increase in degree of dissociation occurs even when there is no change in ionization state of the phosphate.

Table II. Summary of Available Data on the Degree of Dissociation (%) of Phosphates[a]

	phosphate chain length ($\bar{n}$)					
	1	2	3	5.7	12.8	20.8
this work	91.6	88.4	81.6	63.4	45.6	38.0
Schindewolf (1953) conductnc and transfrnc		100.0	97.1	70.3	50.5	42.7
Schindewolf and Bonhoeffer (1953) ion-sel membr	100.0	90.3	83.5	60.2	40.4	34.0
Wall and Doremus (1954) conductnc and transfrnc	95.4	85.1	80.9			
Batra (1965) ion-sel electr	100.0	62.0	60.0	48.0	44.0	
	100.0[b]	90.0[b]	86.0[b]	70.0[b]	61.0[b]	

[a] At concentrations between 10^{-3} and 10^{-2} M. [b] At a concentration of 10^{-4} M.

Effect of Phosphate Chain Length. In the analysis of variance of the data, phosphate chain length was the only variable that had a significant effect ($p < 0.001$) on the degree of dissociation of the phosphates. Because of this result, the degree of dissociation values presented in Table II has been averaged over the four concentrations studied. For comparison, the results obtained in earlier studies by Wall and Doremus (1954), Schindewolf (1953), Schindewolf and Bonhoeffer (1953), and Batra (1965) have also been presented in this table. In addition, the following polynomial, which describes the degree of dissociation of the phosphates in terms of their number-average chain length, was fitted to the data ($R^2 = 98.7\%$):

$$\text{degree of dissocn (\%)} = 98.8 - 6.60(\bar{n}) + 0.178(\bar{n})^2$$

The decrease in degree of dissociation of the phosphates with increasing number-average chain length is similar to that reported in previous studies (see Table II). The results obtained in our study agree best with the results obtained by Schindewolf and Bonhoeffer (1953). This is not completely unexpected as the phosphate concentration, chain length, and method of analysis used in our study were similar to those used in the earlier study. The major difference in results between the two studies is that in our study the degree of dissociation values for the longer chain phosphates (those with $\bar{n} > 3$) were slightly higher than those reported earlier by Schindewolf and Bonhoeffer (1953). This was probably because in our study the concentration of the longer chain phosphates was 10 times lower than in the earlier study. In the earlier study, all phosphates were studied at a fixed molar concentration of 10^{-2} M whereas in our study the phosphates were studied at fixed weight concentration. As a result, in our study, the molar concentration of the shorter chain phosphates was approximately 10^{-2} M, but because the longer chain phosphates had higher molecular weights they had lower molar concentrations.

The degree of dissociation of disodium phosphate reported here (91.6%) was lower than the value of 100% reported in two of the previous studies listed in Table II. The evidence, however, tends to support our finding that orthophosphate is not completely dissociated at the concentrations we studied. Our data indicate that approximately 8.4% of the sodium is not free in solution but is present as an ion pair. The most probably form of the ion pair is NaH_2PO_4 because the ion pair $NaHPO_4^-$ does not form at concentrations similar to those (10^{-4} M) used in this study (Gardner and Nancollas, 1969). This value of 8.4% undissociated sodium is consistent with the values of 4.6% and 7.0% obtained at comparable concentrations by Wall and Doremus (1954) and Smith and Alberty (1956), respectively. Furthermore, other alkali metals such as potassium and lithium form ion pairs with orthophosphate at phosphate concentrations similar to those used in this study (Van Wazer and Callis, 1958).

The range of results shown in Table II highlights the problems associated with measuring the dissociation constants of phosphates at relatively high concentrations. The results of Schindewolf (1953) and Wall and Doremus (1954) illustrate the inaccuracies of conductance–transference methods for determing the extent of dissociation of asymmetric molecules such as phosphates at high concentrations (as has been previously pointed out by Johnson and Pytkowicz (1979)). The results obtained by Batra (1965) illustrate the problems associated with using ion-selective electrodes to determine the degree of ionization of salts without correcting for the effect that ionic strength has on the activity of the ion being measured (Gardner and Nancollas, 1969).

CONCLUSION

Although the results obtained in this study do not differ greatly from those obtained by Schindewolf and Bonhoeffer (1953), they do resolve the problem of conflicting results previously reported in the literature (Schindewolf, 1953; Wall and Doremus, 1954; Batra, 1965). In view of the inaccuracies in many of the previous studies, the degree of dissociation values reported here appear to be the most appropriate for calculating the ionic strength of phosphates used in food products. This is not only because the values reported in this study are accurate but also because the types and weight concentrations of phosphates studied are similar to those used in meat products.

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Registry No. Disodium phosphate, 7558-79-4; tetrasodium pyrophosphate, 7722-88-5; sodium tripolyphosphate, 7758-29-4; sodium tetrapolyphosphate, 14986-84-6; sodium hexametaphosphate, 10124-56-8; sodium phosphate, 7632-05-5.

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Toxicological Properties of *O,S,S*-Trialkyl Phosphorodithioates

Fouad A. F. Ali, Amechi C. Chukwudebe, and T. Roy Fukuto*

A limited series of *O,S,S*-trialkyl phosphorodithioates was prepared. Rat oral toxicities varied widely with very minor changes in alkyl substitutents, ranging from moderately to highly toxic. The order of toxicity to the housefly was different from that of rats. All the compounds were poor in vitro inhibitors at rat erythrocyte cholinesterase, with k_i values ranging from 2 to 177 M^{-1} min^{-1}. The poor anti-AChE activity of these compounds when contrasted with the cholinergic symptoms of poisoning elicited in the rat and housefly suggests that bioactivation may play a significant role in their toxicity. *O,S,S*-Trimethyl phosphorodithioate was several-fold more reactive, both chemically and biochemically, than its corresponding phosphorothioate analogue *O,O,S*-trimethyl phosphorothioate.

INTRODUCTION

Previous reports from this and other laboratories have described the delayed toxic action of *O,O,S*-trimethyl phosphorothioate (OOS-Me) and *O,S,S*-trimethyl phosphorodithioate (OSS-Me). These compounds are present as impurities in such "safe" organophosphorus insecticides as malathion, phenthoate, and acephate (Umetsu et al., 1977; Umetsu et al., 1981; Hammond et al., 1982; Aldridge et al., 1979). Examination of a series of *O,O,S*-trialkyl phosphorothioates related to OOS-Me revealed several other esters with delayed toxic activity, with highest activity found with compounds containing small alkyl groups (Ali and Fukuto, 1982). Some of these compounds were acutely highly toxic to the rat, with oral LD_{50} values ranging from 11 to about 50 mg/kg. Typical signs of poisoning were weight loss, diarrhea, incontinence, and bleeding from the nose or mouth (Gray and Fukuto, 1984; Mallipudi et al., 1979; Umetsu et al., 1981).

The phosphorodithioate ester OSS-Me also displayed toxicological properties similar to OOS-Me at low doses (near the LD_{50} dose of 26 mg/kg), but at high doses ($10LD_{50}$) the signs of poisoning indicated a cholinergic mechanism of intoxication (Aldridge et al., 1979). In an effort to develop additional information on the toxicological properties of compounds of this type, a small series of lower alkyl *O,S,S*-trialkyl phosphorodithioates was synthesized and evaluated for toxicity to rats, anticholinesterase activity, and chemical reactivity. This report presents the results of this study.

MATERIALS AND METHODS

Chemicals. *O,S,S*-Trimethyl phosphorodithioate (**1**) was prepared according to Aldridge et al. (1979). The remaining *O,S,S*-trialkyl phosphorodithioates (**2–6**) were synthesized by reaction between the appropriate alkyl phosphorodichloridate and the sodium alkanethiolate. Typically, the sodium alkanethiolate was added as a powder to a chilled solution of alkyl phosphorodichloridate in anhydrous benzene, followed by heating of the mixture to reflux. After filtration through filter aid, the product was distilled under reduced pressure and subsequently purified by preparative thin-layer chromatography (TLC), using benzene–ethyl acetate (1:1 by volume) as the developing solvent and silica gel GHLF (Analtech, Inc., Newark, DE) as the stationary phase. Structures were verified by NMR and elemental analyses (see Table I). Product purity was determined first by analytical TLC using 1:1 benzene–ethyl acetate. Spots on TLC plates were detected by ultraviolet light and by spraying with 0.75% 2,6-dibromoquinone-4-chloroimide (DBQ) in ether (Menn et al., 1957). Purity was verified further by GLC as described under analysis.

O,O,S-Trimethyl phosphorothioate (**7**) was available from an earlier study (Mallipudi et al., 1979).

Toxicological Evaluation. Acute and delayed rat toxicity was determined with 200–250-g female albino rats (Sprague-Dawley derived) obtained from Simonsen Laboratories, Gilroy, CA. Solutions of the toxicants in corn oil were administered orally at 0.1 mL/100 g to animals fasted for about 12 h before treatment. The rats were kept under observation for 20 days. At least five different doses with a minimum of four rats per dose were used to determine LD_{50} values.

Insecticidal activity was determined with the susceptible S_{NAIDM} strain of houseflies, *Musca domestica*, at 72 °F according to March and Metcalf (1949). LD_{50} values were based on 24-h mortality by using five dosages per compound.

Acetylcholinesterase Inhibition. Rat red blood cell acetylcholinesterase (RBAChE) was prepared by taking 50 μL of blood from a cut in a rat tail and washing the erythrocytes three times with one 10-mL and two 4-mL portions of 0.1 M pH 7.4 sodium phosphate buffer using a Clay Adams desk top centrifuge set at 2000g, for 10, 5, and 5 min, respectively.

The washed erythrocyte pellets were resuspended in 2 mL of buffer, and 0.6 mL of this suspension was diluted

Division of Toxicology and Physiology, Department of Entomology, University of California, Riverside, California 92521.

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