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IDENTIFICATION OF SOME SOLUBLE ESTERASES OF THE CARROT (*DAUCUS CAROTA* L.)*

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Abstract—Aqueous-extractable esterases of the carrot (*Daucus carota* L.) showed optimal activity between pH 6·8 and 7·2. Acetyl, propionyl, and *n*-butyryl esters of phenol, sodium 2-naphthol-6-sulfonate, and glycerol and *n*-hexyl ester of sodium 2-naphthol-6-sulfonate were hydrolyzed by carrot esterases. Lack of activity with *n*-octyl ester of sodium 2-naphthol-6-sulfonate and triolein indicated the absence of a lipase or an esterase able to hydrolyze long-chain soluble esters. Esterases capable of hydrolyzing acetyl-, propionyl-, and *n*-butyrylcholine also were absent in the carrot extract. Carrot esterases showed maximal activity with phenyl esters, while the esters of sodium 2-naphthol-6-sulfonate and triglycerides were hydrolyzed at slower rates. Activity decreased as the acyl chain-length was increased. Inhibition studies with diethyl *p*-nitrophenyl thiophosphate (parathion), tetraethyl pyrophosphate (TEPP) and diisopropylphosphorofluoridate (DFP) at concentrations from 10^{-1} to 10^{-10} M with nine substrates indicated the presence of six esterases. Four esterases were classified as carboxylesterases (EC 3.1.1.1) and two fit into the arylesterase (EC 3.1.1.2) classification. Evidence is presented suggesting the hydrolysis of TEPP and DFP by the carrot extract.

INTRODUCTION

RECENT reports from this laboratory have shown that the pea[1] (*Pisum sativum* L.) and the green bean[2] (*Phaseolus vulgaris* L.) contain complex systems of esterases, which were dissimilar in regard to the number of esterases present and substrate and inhibitor specificities. Both of these vegetables belong to the Leguminosae and the tissues examined contain chlorophyll. These differences in esterases of two similar vegetables led to the present investigations of a completely different vegetable, the carrot (*Daucus carota* L.). Carrots are roots of plants of the Umbelliferae which contain β-carotene as the major pigment. On the basis of substrate and inhibitor specificity, six esterases were identified in aqueous extracts of the carrot. Evidence was also obtained for hydrolysis of certain organophosphorus compounds by enzymes of the carrot.

RESULTS

Effect of pH

To select a suitable pH for use throughout this study, esterase activity toward phenylpropionate (PP), tripropionin (TP), and propionyl ester of sodium 2-naphthol-6-sulfonate (NP) was determined at various pH levels from 6·0 to 8·0. The pH optima was found to lie between 6·8–7·2. A pH of 7·2 was chosen for use in this study. TP hydrolyzing-activity was more sensitive to changes in pH, with lower activity noted at pH 6, and 8, than PP and NP hydrolyzing-activity. pH optima for green bean esterases was reported at 7·2[2] and that of pea esterases at 7·0.[1]

* Technical Paper No. 2417, Oregon Agricultural Experiment Station.

[1] M. J. NORGAARD and M. W. MONTGOMERY, *Biochem. Biophys. Acta* **151**, 587 (1968).
[2] T. B. PUTNAM and M. W. MONTGOMERY, *J. Food Sci.*, in press.

Substrate Specificity

Activities of carrot esterases toward the various substrates are shown in Table 1. With these substrates, carrot extracts possessed approximately 10 per cent the activity of similarly prepared extracts from the pea[1] and the green bean.[2] These results agree with those of Schwartz *et al.*,[3] who reported that carrot had approximately 25 per cent the esterase activity of green bean. Phenyl esters were hydrolyzed most rapidly, followed by naphthyl esters and triglycerides. No activity was noted with choline esters. Similar results were obtained on the pea[1] and green bean.[2] The most non-polar substrates (phenyl esters) were attacked at the fastest rates, while the most polar substrates (choline esters) were hydrolyzed at the slowest rate, if at all. Therefore, non-polar groups were important in binding of the substrate to the enzyme.[4]

TABLE 1. HYDROLYSIS OF VARIOUS ESTERS BY CARROT EXTRACT

Substrates	Activity (units)*	Replications	Standard deviation (±)
Phenyl acetate	0·89	8	0·11
Phenyl propionate	0·89	4	0·03
Phenyl *n*-butyrate	0·75	5	0·07
Acetyl ester of sodium 2-naphthol-6-sulfonate	0·47	3	0·02
Propionyl ester of sodium 2-naphthol-6-sulfonate	0·34	3	0·02
n-Butyryl ester of sodium 2-naphthol-6-sulfonate	0·16	3	0·01
n-Hexyl ester of sodium 2-naphthol-6-sulfonate	0·10	3	0·00
n-Octyl ester of sodium 2-naphthol-6-sulfonate	0·00	3	0·00
Triacetin	0·16	3	0·01
Tripropionin	0·15	3	0·00
Tri-*n*-butyrin	0·09	3	0·01
Triolein	0·00	3	0·00
Acetylcholine iodide	0·00	3	0·00
Propionylcholine iodide	0·00	3	0·00
n-Butyrylcholine iodide	0·00	3	0·00

* One activity unit represents one microequivalent of substrate hydrolyzed/min/ml of carrot extract.

As the acyl chain-length of the substrate was increased, the rate of hydrolysis decreased. This is contrasted by the results of the pea[1] and green bean,[2] which showed that the propionyl esters were hydrolyzed faster than either acetyl, or *n*-butyryl, esters. Lack of activity with triolein and the soluble longer-chain esters of sodium 2-naphthol-6-sulfonate suggests the absence of a lipase[5] or esterases in the carrot extract capable of hydrolyzing longer-chain esters. Similar results indicated the absence of lipase from an aqueous extract of the pea[1] and green bean.[2] However, the presence of lipase has been reported in plant tissue,[6] suggesting that in the present study, the lipase might not have been extracted and/or was not active under the experimental conditions used.

[3] H. M. SCHWARTZ, S. I. BEIDRON, M. M. VON HOLDT and S. REHM, *Phytochem.* **3**, 189 (1964).
[4] M. DIXON and E. C. WEBB, *Enzymes*, 2nd edition, p. 220, Academic Press, New York (1964).
[5] P. DESNUELLE and P. SAVARY, *J. Lipid Res.* **4**, 369 (1963).
[6] F. A. LEE and A. C. WAGENKNECHT, *Food Res.* **23**, 584 (1958).

Effect of EDTA

Norgaard and Montgomery[1] reported inhibition of pea esterases by bivalent metallic ions, while metal complexing agents (EDTA, cysteine-HCl, and ethyl mercaptan) were activators. Also, less variance in the activity in the presence of EDTA was noted, which was attributed to the presence of metallic ions in distilled water. Although water distilled in an all-glass apparatus was used in this study, it was desirable to study the effect of EDTA on the activity of carrot esterases.

Data presented in Table 2 indicate that EDTA inhibited esterase activity at the higher concentrations and caused a slight, but inconsistent, activation at lower concentrations. This slight activation did not warrant the inclusion of EDTA in the reaction mixture.

TABLE 2. EFFECT OF EDTA ON ESTERASE ACTIVITY OF CARROT

EDTA concentration (mM)	Phenyl propionate	Tripropionin	Propionyl ester of sodium 2-naphthol-6-sulfonate
	% Inhibition*		
25	33	46	18
15	27	24	11
5·0	15	10	10
2·5	8	2	1
1·0	6	−2	−9
0·1	3	2	−15
0·025	3	1	4
0·005	−1	3	−3
0·00025	1	7	17

* Values shown are means of three determinations.

Inhibitor Specificity

Inhibition patterns of carrot esterases by various concentrations of diethyl *p*-nitrophenyl thiophosphate (parathion), diisopropylphosphorofluoridate (DFP), and tetraethyl pyrophosphate (TEPP) are presented in Figs. 1–3, respectively. In general, sigmoid curves were obtained. A single sigmoid curve would indicate the presence of one enzyme active toward the substrate, while a double sigmoid curve would suggest two enzymes hydrolyzed the substrate. However, esterases having similar substrate and inhibitor specificities would not be differentiated.

Curves presented in Fig. 1A indicate the presence of three esterases, which hydrolyzed phenyl esters. The most sensitive esterase was inhibited between pI (negative $\log_{10}$ of the molar inhibitor concentration) 7 and 4 and showed activity with all phenyl esters. This esterase accounted for most of the phenyl *n*-butyrate (PB) hydrolyzing activity and approximately 75 per cent of the phenyl acetate (PA) and PP hydrolyzing activity. Differences in inhibition at pI 4 demonstrated the presence of the second esterase, which was inhibited between pI 4 and 1. This second esterase was more active toward PP than PA, as revealed by the greater inhibition of PP hydrolysis compared to PA hydrolysis. The second esterase was not active toward PB. Activity toward PA, which was not inhibited at pI 1 of parathion, suggests the presence of the third esterase resistant to parathion. This shows some similarity to an esterase in the pea, which was specific for PA and resistant to parathion.[1] The pea esterase was, however, less resistant than the carrot esterase.

The data presented in Fig. 1B also suggest the presence of three esterases, which hydrolyzed the triglycerides and were similar to those mentioned above. The hydrolysis of tri-*n*-butyrin (TB) was most sensitive to inhibition, while the hydrolysis of triacetin (TA) was more resistant.

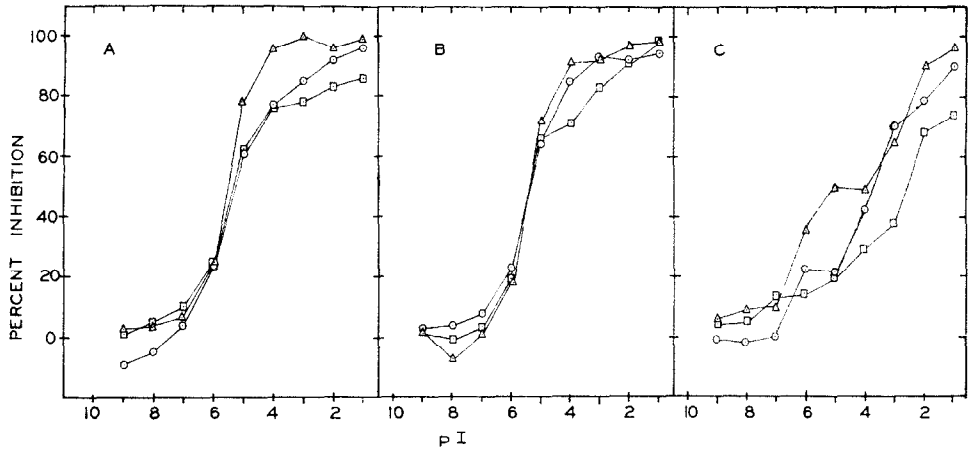

FIG. 1. INHIBITION BY VARIOUS CONCENTRATIONS OF PARATHION OF THE HYDROLYSIS OF A, PHENYL ESTERS; B, TRIGLYCERIDES; AND C, ESTERS OF SODIUM 2-NAPHTHOL-6-SULFONATE. pI IS THE NEGATIVE $\log_{10}$ OF THE MOLAR INHIBITOR CONCENTRATION.

Legend: □—□ acetyl esters; ○—○ propionyl esters; △—△ butyryl esters.

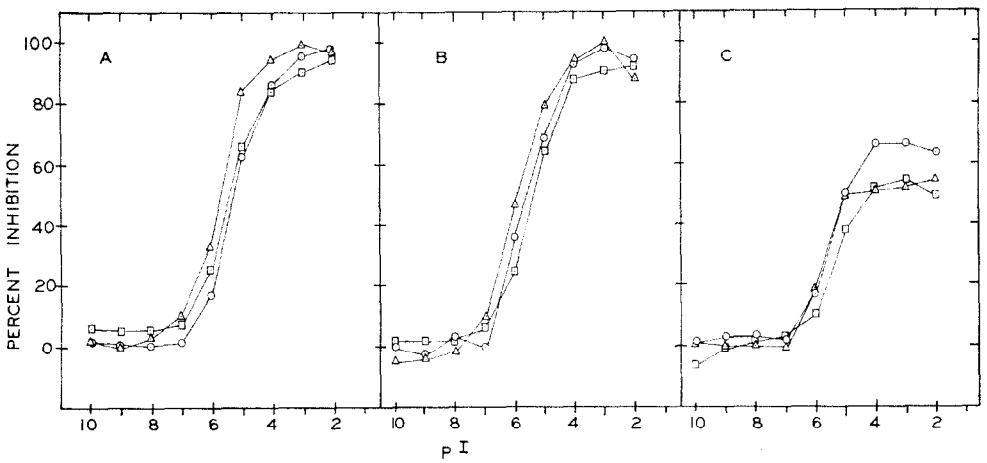

FIG. 2. INHIBITION BY VARIOUS CONCENTRATIONS OF DFP OF THE HYDROLYSIS OF A, PHENYL ESTERS; B, TRIGLYCERIDES; AND C, ESTERS OF SODIUM 2-NAPHTHOL-6-SULFONATE. pI IS THE NEGATIVE $\log_{10}$ OF THE MOLAR INHIBITOR CONCENTRATION.

Legend: □—□ acetyl esters; ○—○ propionyl esters; △—△ butyryl esters.

The esterase resistant to pI 1 of parathion appeared to attack TP more readily than TB and TA.

Study of the curves in Fig. 1C indicates that carrots contain more than three esterases. Here, the inhibition by parathion between pI 7 and 4 suggests the presence of three esterases. One was inhibited between pI 7 and 6 and active toward NP and *n*-butyryl ester of sodium

2-naphthol-6-sulfonate (NB). The second esterase was active toward NB and inhibited between pI 6 and 5, but, since no inhibition of NP hydrolysis occurred at this concentration of parathion, this esterase was not active with NP. The third esterase was inhibited between pI 5 and 4 and was active toward NP but not NB. These esterases were active toward the acetyl ester of sodium 2-naphthol-6-sulfonate (NA), as indicated by some inhibition of NA hydrolysis between pI 7 and 4. It is assumed that one or all of these esterases were responsible for the activity toward phenyl esters and triglycerides. Between pI 4 and 1, a fourth esterase was inhibited, which might have been the same esterase as the one inhibited at this concentration of parathion in Figs. 1A and 1B. Since inhibition did not reach 100 per cent, particularly with NA, the presence of a fifth esterase is revealed. This esterase was more active toward NA than NP and NB, and probably was the same esterase which was resistant to parathion and hydrolyzed PA (Fig. 1A).

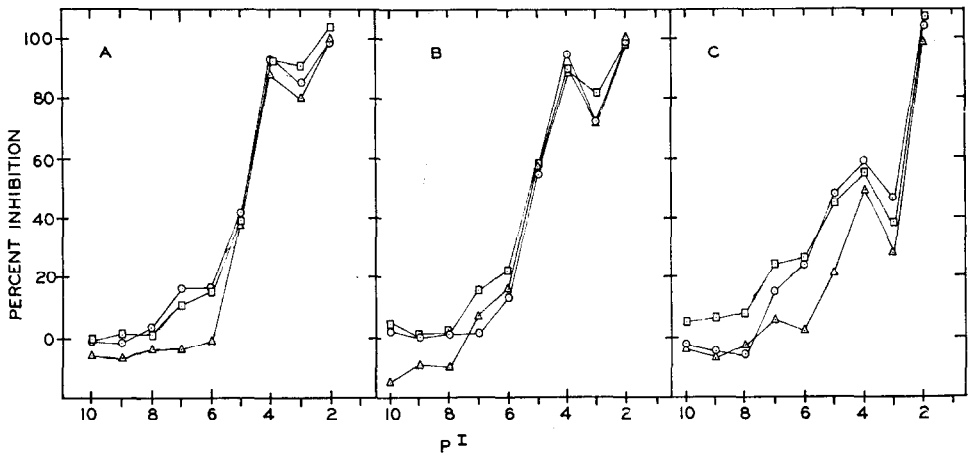

FIG. 3. INHIBITION BY VARIOUS CONCENTRATIONS OF TEPP OF THE HYDROLYSIS OF A, PHENYL ESTERS; B, TRIGLYCERIDES; AND C, ESTERS OF SODIUM 2-NAPHTHOL-6-SULFONATE. pI IS THE NEGATIVE $\log_{10}$ OF THE MOLAR INHIBITOR CONCENTRATION.

Legend: □—□ acetyl esters; ○—○ propionyl esters; △—△ butyryl esters.

Figures 2A and 2B show that the inhibition of hydrolysis of phenyl esters and triglycerides by DFP approached 100 per cent. Only single sigmoid curves were obtained, indicating that DFP was not as selective an inhibitor as parathion. Analogous to parathion, the hydrolysis of PB and TB was more sensitive to DFP. Activity toward the naphthyl esters was not completely inhibited by DFP (Fig. 2C) which demonstrated that an esterase resistant to DFP was present in carrot extract. Since only slight activity was noted with PA (Fig. 2A) and a greater percentage of naphthyl ester hydrolyzing activity was resistant to DFP (Fig. 2C) than parathion (Fig. 1C), it would appear that this sixth esterase was not the same as the parathion-resistant esterase (Figs. 1A and 1C). Putnam and Montgomery[2] also reported a DFP-resistant esterase specific for naphthyl esters in green bean.

Figure 3A reveals the presence of two esterases, which hydrolyzed phenyl esters and were inhibited by TEPP. The most sensitive esterase was inhibited between pI 8 and 7. Absence of inhibition of PB hydrolysis indicates that the most sensitive esterase was not active with PB. The less sensitive esterase was inhibited between pI 6 and 4 and was active toward all phenyl esters. These two esterases were probably the same two described above (Fig. 1A)

that were sensitive to parathion. The esterase, which was resistant to parathion and hydrolyzed PA, was inhibited by TEPP.

Figure 3B shows the presence of an esterase active with TP and TA, but not toward TB, as indicated by absence of inhibition of TB hydrolysis between pI 8 and 7. The esterase active toward the three triglycerides was inhibited between pI 7 and 4. There was no evidence to show that these two esterases were different from the esterases shown in Fig. 3A. Comparison of the inhibition toward naphthyl esters at pI 4, with that of other esters, indicates that esterases hydrolyzing naphthyl esters were more resistant to TEPP (Fig. 3C). Also activity with NB was more resistant to TEPP than the activity with NA and NP.

It is apparent from Fig. 3 that inhibition with TEPP did not show a definite sigmoid curve because of the decrease in inhibition at pI 3. Figures 3A and 3B show that inhibition

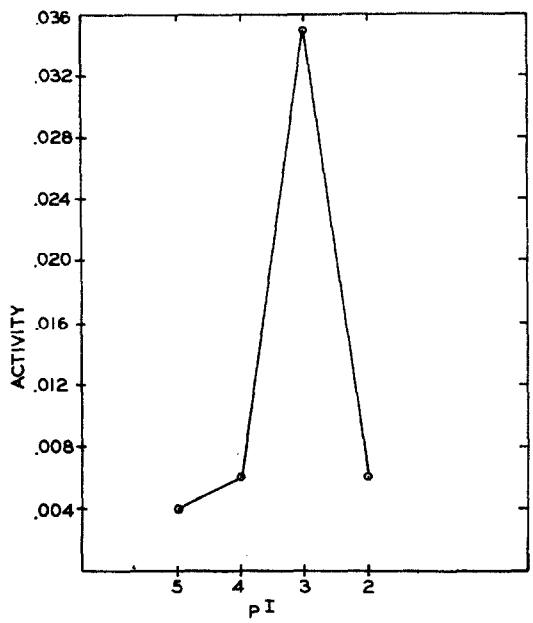

FIG. 4. EFFECT OF TEPP CONCENTRATION ON THE HYDROLYSIS OF TEPP BY CARROT EXTRACT. pI IS THE NEGATIVE $\log_{10}$ OF THE MOLAR INHIBITOR CONCENTRATION. EACH POINT IS THE MEAN OF FIVE DETERMINATIONS.

approached 100 per cent at pI 4, decreased considerably at pI 3, and increased to approximately 100 per cent at pI 2. This dip is very prominent in Figs. 3B and 3C. To investigate this phenomenon, assays were run at different concentrations of TEPP in the absence of substrate. A solution of 3 per cent (w/v) Triton X-155 and 0·1 per cent (w/v) gum arabic replaced the substrate. Enzyme extracts were undiluted.

Results (Fig. 4) show that very little activity was noted, except at pI 3. Decrease in inhibition at pI 3 (Fig. 3) might be explained by the comparatively high TEPP-hydrolyzing activity at pI 3. At concentrations less than pI 3, there was not sufficient TEPP to serve as substrate for the enzyme. At pI 2, TEPP might have inhibited both the TEPP-hydrolyzing enzyme and the esterases, which hydrolyzed the substrate. Inhibition of the TEPP-hydrolyzing enzyme could have been by excess TEPP at pI 2 or by the presence of impurities in the TEPP. Since pure TEPP was not available, commercial grade TEPP was used, which con-

tained 40 per cent TEPP and 60 per cent other ethyl phosphates. At pI 2, the concentration of other ethyl phosphates might have been sufficient to inhibit both the TEPP-hydrolyzing enzyme and esterases hydrolyzing the substrate. The presence of an enzyme-hydrolyzing DFP is suggested in Fig. 2B by the decrease in inhibition of the hydrolysis of TB from pI 3 to 2. No evidence is available to determine if the DFP-hydrolyzing enzyme was the same as the TEPP-hydrolyzing enzyme. The DFP-resistant esterase, noted in Fig. 2C, and the TEPP-resistant esterase, Fig. 3C, may have been responsible for the activity toward these organophosphorus compounds. Forster *et al.*[7] also noted a decrease of inhibition with bovine milk esterases at higher concentrations of organophosphorus compounds. This was later attributed to hydrolysis of organophosphorus inhibitors by arylesterase of milk.[8] The hydrolysis of certain organophosphorus inhibitors by arylesterases from vertebrate blood plasma has also been reported.[9]

DISCUSSION

Six esterases were shown to be present in the aqueous extract of lyophilized carrots. There exists the possibility that more esterases were present and were not revealed due to similar substrate and inhibitor specificities. Esterases reported in this study were not isozymes, since they were differentiated on the basis of substrate and inhibitor specificities.

Animal esterases have been classified with regard to substrate and inhibitor specificities.[9,10] Carboxylesterases (carboxyl ester hydrolases, EC 3.1.1.1), formerly known as aliesterases or B-esterases, are those enzymes which hydrolyze both aliphatic and aryl esters, but not esters of choline. Carboxylesterases are inhibited by most organophosphorus inhibitors, but not physostigmine. Arylesterases (aryl ester hydrolases, EC 3.1.1.2), formerly known as A-esterases, are not inhibited by most organophosphorus inhibitors or physostigmine, but are inhibited by *p*-hydroxymercuribenzoate. Arylesterases hydrolyze aromatic esters, but normally not aliphatic esters.

Plants have also been shown to contain a variety of esterases. Jansen *et al.*[11] reported the presence of an acetylesterase (acetic ester acetyl-hydrolase, EC 3.1.1.6) in citrus fruits, which hydrolyzed esters of acetic acid more rapidly than esters of higher homologs and was inhibited by DFP. Using starch-gel electrophoresis and various substrates and inhibitors, Jooste and Moreland[12,13] demonstrated complex systems of esterases (both carboxylesterases and arylesterases) in cucumber, wheat seed, soybean and corn. One esterase, specific for 2-naphthol-phenoxyacetate, was activated by DFP.[13] With similar techniques, Schwartz *et al.*[3] observed several esterases in green bean, cabbage, potato tuber, citrus albedo and flavedo and fruits of many cucurbits. These esterases were shown to vary between species, within strains of the same species, and in different parts of the same plants.[3]

Six bands of esterase activity were separated by starch-gel electrophoresis from extracts of twelve varieties of germinating peas.[14] Six esterases were also revealed in the pea by differentiation on the basis of inhibitor and substrate specificities.[1] Five of these esterases

[7] T. L. FORSTER, H. A. BENDIXEN and M. W. MONTGOMERY, *J. Dairy Sci.* **42**, 1903 (1959).
[8] T. L. FORSTER, *J. Dairy Sci.* **44**, 1164 (1961).
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[12] J. VAN DER W. JOOSTE and D. E. MORELAND, *Phytochem.* **2**, 263 (1963).
[13] J. VAN DER W. JOOSTE and D. E. MORELAND, *Nature* **195**, 907 (1962).
[14] T. A. FRANKEL and E. D. GARBER, *Botan. Gaz.* **126**, 221 (1965).

were classified as corboxylesterases, while the other, which was resistant to DFP, was classified as an arylesterase. A DFP-resistant esterase was reported present in the green bean and was tentatively classified as an arylesterase.[2] Two other esterases in the green bean, both inhibited by organophosphorus inhibitors, were classified as carboxylesterases.[2]

In the present study, four of the six esterases found in the carrot extract were sensitive to the organophosphorus inhibitors and would fit into the carboxylesterase classification. However, some of these esterases showed more activity with the aromatic substrates than the aliphatic substrates (Table 1). The esterase, resistant to parathion, but inhibited by DFP and TEPP, and hydrolyzed PA (Fig. 1A), but had only slight activity with the triglycerides, could fit into the arylesterase classification. Similarly, the DFP- and TEPP-resistant esterase, which hydrolyzed the naphthyl esters, but not phenyl esters or triglycerides, would be classified as an arylesterase. As mentioned earlier, the enzyme hydrolyzing the organophosphorus inhibitors might be one of the esterases resistant to these inhibitors and would fit the classification of an arylesterase. More research is required on purified enzyme preparations to defined the substrate and inhibitor specificities of these esterases precisely and to determine a more precise classification. It would be wise to delay proposing nomenclature for these esterases until such research is completed. Investigations to obtain this information are currently under way in this laboratory.

MATERIALS AND METHODS

Freshly harvested carrots (*Daucus carota* L., variety Imperada) were peeled, diced, lyophilized, sealed in cans in an atmosphere of nitrogen, and stored at $-18°$. The dry carrot tissue was powdered in a Waring Blendor for 2 min at maximum speed. A slurry of 10 parts water and 1 part powdered carrot (v/w) was centrifuged in the cold (3°) at $31{,}000 \times g$ for 25 min. The supernatant was filtered through glass wool and appropriate dilutions were used as enzyme preparations. For the control, enzyme preparations were heated in a boiling water bath for 10 min and filtered through Whatman No. 12 filter paper to remove the precipitate.

Substrates were prepared as described by Norgaard and Montgomery.[1] The following substrates were used: PA, PP, TA, TP, TB, and acetylcholine iodide from Eastman; PB, triolein, propionylcholine iodide, and *n*-butyrylcholine iodide from K & K laboratories; and NA, NP, NB, and *n*-hexyl and *n*-octyl esters of sodium 2-naphthol-6-sulfonate from T. L. Forster, Washington State University.

Organophosphorus inhibitors were prepared as previously described.[1] Inhibitors included in this study were parathion and DFP from Eastman and TEPP from California Chemical Co. TEPP was commercial grade and contained 40 per cent TEPP and 60 per cent other ethyl phosphates.

Manometric assay procedure for carrot esterases was identical to that described in a previous publication.[1]

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