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PRELIMINARY CHARACTERIZATION OF SOME PLANT CARBOXYLIC ESTER HYDROLASES

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Abstract—Carboxylic ester hydrolase preparations were obtained from wheat seeds (*Triticum aestivum* L. "Anderson") and from acetone powders of 5- to 7-day-old cucumber (*Cucumis sativus* L. "Stono"), soybean (*Glycine max* L. Merr. "Lee"), and corn (*Zea mays* L. "GT 112×CI. 21") seedlings. Evidence obtained from electrophoretic mobilities, substrate specificities, and inhibitor studies indicated that the four species of plants contained complex systems of ester hydrolases. Ester hydrolases were partially and tentatively identified with criteria established for animal serum enzymes.

INTRODUCTION

TERMINOLOGY associated with fatty-acid ester hydrolases, enzymes which catalyze the hydrolysis of fatty-acid esters, is confusing. The confusion has resulted from the indiscriminate use of various terms and the overlapping specificities of some of the enzymes. Hofstee[1] attempted to clarify the situation and suggested that fatty-acid ester hydrolases be divided into (a) enzymes which act on substrates in solution (ester hydrolases proper) and (b) enzymes which act predominantly on undissolved substrates (lipase-type ester hydrolases). The term "lipase" was reserved by Hofstee for the glycerol ester hydrolases (fat-splitting enzymes).

Ester hydrolases proper (from blood plasma) have been divided into A- and B-ester hydrolases [2-4] on the basis of their response to organophosphorous inhibitors. B-ester hydrolases (carboxylic ester hydrolases or "ali-esterases") are inhibited by organophosphorous chemicals, whereas A-ester hydrolases (arylester hydrolases or arylesterases) are not. A-ester hydrolases are not inhibited because they hydrolyze organophosphorous inhibitors such as diethyl *p*-nitrophenyl phosphate (E-600)[5] and diisopropylfluorophosphate (DFP).[6] B-ester hydrolases readily hydrolyze both aliphatic and aromatic esters of short-chain fatty acids, whereas A-ester hydrolases hydrolyze only aromatic esters.[3,4] B-ester hydrolases have been further subdivided into acetyl-, propionyl-, and butyryl-ester hydrolases in accordance with their substrate specificities.[3,4] Bergmann *et al.*[7] reported that A-ester hydrolases are inhibited by *p*-chloromercuribenzoate (PCMB). They also described a C-ester hydrolase in hog kidney extracts which behaved like an A-ester hydrolase, but was stimulated by PCMB at concentrations which inhibited A-ester hydrolases. Although classified as ester hydrolases proper, cholinesterases are inhibited by 10^{-5} M physostigmine, whereas A- and B-ester hydrolases are not.[3,4]

Few attempts have been made to correlate properties and characteristics of plant ester

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[1] B. H. J. HOFSTEE, *The Enzymes*, (Edited by P. D. BOYER, H. LARDY and K. MYRBÄCK) Vol. 4, p. 485, Academic Press Inc., New York (1960).
[2] W. N. ALDRIDGE, *Biochem. J.* **53**, 110 (1953).
[3] K. B. AUGUSTINSSON, *Nature* **181**, 1786 (1958).
[4] K. B. AUGUSTINSSON, *Acta Chem. Scand.* **13**, 571 (1959).
[5] W. N. ALDRIDGE, *Biochem. J.* **53**, 177 (1953).
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hydrolases with their animal counterparts. An ester hydrolase from citrus[8] and wheat germ esterase[9] were originally described as acetylesterases. Both enzymes are inhibited by DFP. A phenoxyester hydrolase (phenoxyesterase) from soybean seedlings differed from enzymes previously described in that it was highly specific for 2-naphthyl phenoxyacetate (NPOA), not inhibited by PCMB, and slightly activated by DFP.[10]

The objectives of the present study were to characterize and identify carboxylic ester hydrolase enzymes in selected plant species with criteria previously established for animal plasma ester hydrolases.

RESULTS

Substrate Specificities

The relative hydrolytic activities of crude ester hydrolase preparations obtained from wheat seeds and from acetone powders of corn, cucumber, and soybean seedlings on 2-naphthyl esters of acetic, butyric, caprylic, and phenoxyacetic acids were compared (Table 1). Enzymes from all four species readily hydrolyzed 2-naphthyl acetate (NA). Consequently,

TABLE 1. RELATIVE HYDROLYTIC ACTIVITIES OF WHEAT, CORN, SOYBEAN, AND CUCUMBER ESTER HYDROLASES ON 2-NAPHTHYL ACETATE, BUTYRATE, CAPRYLATE, AND PHENOXYACETATE AT PH 7·0 AND 37°C

Source of ester hydrolase	2-naphthyl ester used as substrate*		
	butyrate	caprylate	phenoxyacetate
Wheat	10	0	68
Corn	12	†	108
Cucumber	90	16	67
Soybean	50	0	150

* Results expressed as per cent relative activity with the hydrolysis of 2-naphthyl acetate assigned the value of 100.

† Not determined.

the NA activity was assigned the value of 100 and activities on other substrates were related to the NA activity. Enzymes of the two monocotyledonous species (corn and wheat) did not hydrolyze 2-naphthyl butyrate (NB) as readily as enzymes of the two dicotyledonous species (soybean and cucumber). Only cucumber enzymes hydrolyzed 2-naphthyl caprylate (NC) under the conditions of the experiments. Enzymes from all four species hydrolyzed NPOA. Soybean enzymes hydrolyzed NPOA more rapidly than NA and corn enzymes also expressed a slightly higher affinity for NPOA than NA.

Effect of Inhibitors

Enzyme preparations were incubated with inhibitors at 37°C and pH 7·0 for 10 min. prior to the addition of the substrate. Physostigmine (10^{-3} M) did not inhibit the activity of enzymes which hydrolyzed NA and NB. Hence, choline esterases probably did not participate in the hydrolysis of the substrates being studied.

Results of studies with DFP are summarized in Table 2. Hydrolytic activity expressed in the presence of the inhibitor was related to activity obtained without the inhibitor and the

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[9] E. F. JANSEN, M-D. F. NUTTING and A. K. BALLS, *J. Biol. Chem.* **175**, 975 (1948).
[10] J. v. d. W. JOOSTE and D. E. MORELAND, *Nature* **195**, 907 (1962).

TABLE 2. EFFECT OF DIISOPROPYLFLUOROPHOSPHATE ON THE ACTIVITY OF PLANT ESTER HYDROLASES

Source of ester hydrolase	Inhibitor concentration (molar)	2-naphthyl ester used as substrate*		
		acetate	butyrate	phenoxyacetate
Wheat	10^{-4}	71	†	49
	10^{-5}	16	†	29
Corn	10^{-3}	19	†	4
Cucumber	10^{-3}	†	100	76
	10^{-4}	†	95	55
	10^{-5}	48	68	27
Soybean	10^{-3}	46	80	14
	10^{-4}	22	45	8
	10^{-5}	9	21	0

* Results expressed as per cent inhibition.
† Not determined.

results are presented in the table as percent inhibition. The enzymes from the various species which hydrolyzed NA showed the following order of decreasing sensitivity to DFP: cucumber > wheat > soybean > corn. Because of the limited hydrolysis of NB by corn and wheat enzymes, the effect of DFP was not determined for this substrate. Enzymes of cucumber and soybean which hydrolyzed NB were strongly inhibited by DFP. NPOA-hydrolyzing enzymes in corn and soybean showed only a limited response to DFP while those in cucumber and wheat showed a marked response.

Results of inhibition studies with 10^{-3} M PCMB are summarized in Table 3. All inhibitions could be reversed with 2,3-dimercaptopropanol (BAL); therefore, the presence of sensitive thiol groups in the affected enzymes is indicated. The amount of inhibition obtained, however, varied with the substrate. The NA-hydrolyzing enzymes of wheat and soybean were less sensitive to PCMB than those of corn and cucumber. Cucumber and

TABLE 3. EFFECT OF 10^{-3} M *p*-CHLOROMERCURIBENZOATE ON THE ACTIVITY OF PLANT ESTER HYDROLASES.

Source of ester hydrolase	2-naphthyl ester used as substrate*		
	acetate	butyrate	phenoxyacetate
Wheat	26	20	64
Corn	53	†	36
Cucumber	64	93	40
Soybean	31	77	5

* Results expressed as per cent inhibition.
† Not determined.

soybean enzymes which hydrolyzed NB were the most sensitive to PCMB. The NPOA-hydrolyzing soybean enzymes were essentially not affected by PCMB, whereas enzymes from the other three species were moderately sensitive.

Results of inhibitor studies performed with crude enzyme preparations are difficult to interpret because an average response is measured. Little information is available relative to those proportions of the total activity contributed by the different species of enzymes. The high concentrations of inhibitors (10^{-3} M) should be sufficient to essentially completely

inhibit sensitive enzymes. If this assumption is made, the values given in the tables can be interpreted to represent that percentage of the total hydrolytic activity contributed by the inhibitor-sensitive enzymes present in the mixed population of ester hydrolases. The results of the inhibitor studies do emphasize the diverse properties possessed by the enzymes from the four species of plants as related to the relative affinities of the enzymes for the different substrates.

Starch-gel Electrophoresis

Starch-gel electrophoresis provided an additional indication of the ester hydrolase complex in cucumber, soybean, and wheat preparations (Figs. 1–3 respectively). Indicated in the figures (reduced by ½) are the appearences of gels after electrophoresis for 15 hr, incubation in a substrate-diazonium salt mixture for 1 hr, and a thorough wash in running tap

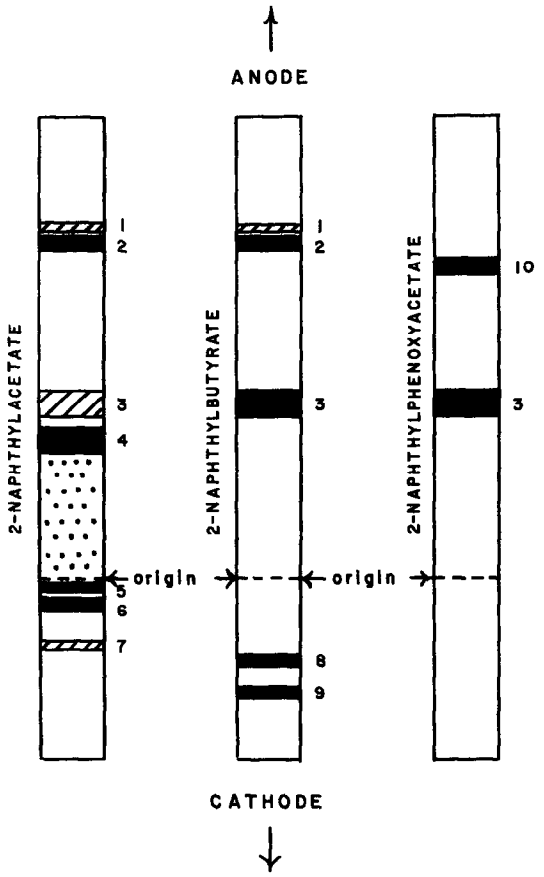

FIG. 1. DIAGRAMMATIC REPRODUCTION OF A ZYMOGRAM OBTAINED WITH CUCUMBER ESTER HYDROLASES

The relative intensity, size, and location of the ester hydrolase-containing regions are shown. Electrophoresis extended for 15 hr at 3°C with a potential gradient of 8 V/cm and 0·025 M borate buffer (pH 8·6). After electrophoresis the gels were incubated for 1 hr in a substrate mixture which contained 100 ml water, 10 ml 0·2 M phosphate buffer pH 7·0, 1 ml of the indicated 2-naphthyl-ester stock solution (1% in 1,4-dioxane), 2 ml Triton X-100, and 100 mg Naphthanil Diazo Blue B. (Legend: ■ intensely stained; ▨ lightly stained; and ⁘ streaked zone.)

water. Criteria employed to evaluate the gel patterns (zymograms) included the rapidity with which the zones became delineated when incubated in the substrate mixture and the final intensity attained by the dye within each band.

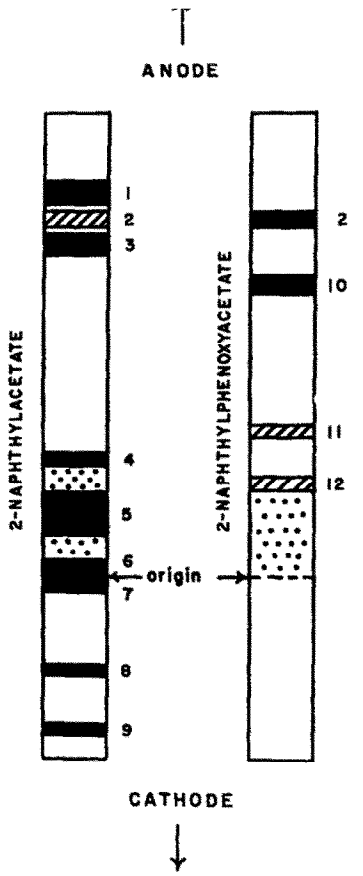

FIG. 2. DIAGRAMMATIC REPRODUCTION OF A ZYMOGRAM OBTAINED WITH SOYBEAN ESTER HYDROLASES
Experimental conditions, as in Fig. 1.

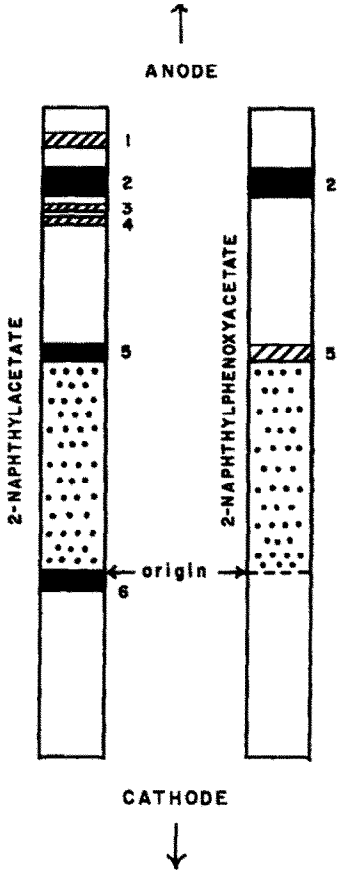

FIG. 3. DIAGRAMMATIC REPRODUCTION OF A ZYMOGRAM OBTAINED WITH WHEAT ESTER HYDROLASES
Experimental conditions, as in Fig. 1.

On the zymograms made with cucumber enzymes, 7 zones contained enzymes which hydrolyzed NA, enzymes in 5 zones hydrolyzed NB, and those in 2 zones hydrolyzed NPOA (Fig. 1). Enzymes in bands 1 and 2 hydrolyzed both NA and NB. Enzymes in band 2 were most active in hydrolyzing NA, i.e., an intense color developed in this area within 2 to 3 min after immersion of the gel in the substrate mixture. Enzymes in band 2 also had the highest affinity for NB; however, their activity was not as great on NB as on NA. Enzymes in band 3 hydrolyzed all three substrates, but showed more affinity for NPOA and NB than for NA. Enzymes in the remainder of the bands hydrolyzed specifically the indicated substrates. Considerable streaking occurred adjacent to the origin with NA as substrate and no distinct bands could be detected in this area. Streaking was less prominent with NB and NPOA.

On the gel electrophoretograms made with soybean preparations, NA-hydrolyzing enzymes were separated into 9 zones and the NPOA-hydrolyzing enzymes into 4 distinct zones (Fig. 2). Zone 2 was the only distinct area which contained enzymes that hydrolyzed both substrates. However, these enzymes manifested a greater affinity for NPOA than for NA. Of the NA-hydrolyzing enzymes, bands 1 and 3 developed first and had the deepest color. Bands 5 and 6 also contained considerable activity. In the presence of NPOA, the intensity of band 10 approached that of band 2. The enzymatic content of the remainder of the bands was either low or the enzymes possessed limited affinity for NPOA.

Gel patterns obtained with wheat enzymes are presented in Fig. 3. NA-hydrolyzing enzymes were separated into 6 distinct bands. An extensive streaked area above the origin was also obtained within which bands were not defined. Enzymatic activity was most intense within band 2 which became evident within 2 to 3 min after immersion of the gel in the substrate mixture and finally attained a very deep color. NPOA-hydrolyzing enzymes were separated into 2 distinct zones in addition to an extensive streaked area adjacent to the origin. All the areas which contained NPOA-hydrolyzing enzymes corresponded with NA-hydrolyzing areas. However, the enzymes expressed a greater affinity for NA than for NPOA.

From a study such as this little information is provided on how the enzymes in the various bands actually differ. Differences in migratory distances can; however, be attributed to such factors as charges, sizes, and shapes possessed by the ester hydrolase molecules and relative degrees of adsorption of the ester hydrolases to the starch. Hence, the enzymes in the various zones must possess different physical properties and electrical charges. More than a single species of enzyme conceivably can be present within a given band, however.

DISCUSSION

Evidence obtained from electrophoretic mobilities, substrate specificities, and action of inhibitors indicated that cucumber, soybean, wheat, and corn contain complex systems of ester hydrolases. In this exploratory study, essentially only the complexity and magnitude of the problem were developed.

All the substrates used in this study were in solution; hence, the ester hydrolases that were studied can be classified as ester hydrolases proper.[1] The presence of glycerol ester hydrolases was not determined.

Cucumber enzymes which hydrolyzed NA and NB were strongly inhibited by DFP (Table 2) and can be considered as B-ester hydrolases under the serum-ester hydrolase classification scheme. Since the NB-hydrolyzing enzymes were more sensitive to PCMB than the NA-hydrolyzing enzymes (Table 3), different enzymes must be involved. Conceivably, acetyl- and butyryl-ester hydrolases were both present. NPOA-hydrolyzing enzymes were less sensitive to both DFP and PCMB than the NA- and NB-hydrolyzing enzymes. However, inhibition by DFP was sufficient to suggest that B-ester hydrolases (Fig. 1, band 3) participated in the hydrolysis of NPOA.

Soybean enzymes, when compared on a relative basis, did not hydrolyze NC, hydrolyzed NB at one-half the extent of NA, and manifested highest activity on NPOA (Table 1). The NB-hydrolyzing enzymes were most sensitive to DFP and PCMB; whereas the NPOA-hydrolyzing enzymes were only slightly inhibited by DFP (Table 2) and PCMB (Table 3). A soybean enzyme which expressed strong affinity for NPOA and was insensitive to DFP and PCMB has been partially-purified and described.[10] The name phenoxyesterase (phenoxyester hydrolase) was suggested for this enzyme. From the differential response to

DFP, some of the enzymes which hydrolyzed NA and NB could be considered to be of the B-type and others of the A-type.

Wheat enzymes actively hydrolyzed NA, were only moderately active on NPOA, produced limited hydrolysis of NB, and did not hydrolyze NC under the experimental conditions (Table 1). The strong inhibition of NA-hydrolyzing enzymes by DFP (Table 2) suggests that acetyl-esterases were present. A similar conclusion was reached by Jansen *et al.*[9] NPOA-hydrolyzing enzymes were less sensitive to DFP and more sensitive to PCMB (Table 3) than the NA-hydrolyzing enzymes. A-ester hydrolases or enzymes of the phenoxyester hydrolase type may have participated in the hydrolysis of NPOA. The limited hydrolysis of NB suggested that butyrylesterases were only minor constituents of the ester hydrolase complex in wheat.

The NPOA-hydrolyzing activity of corn ester hydrolases was slightly higher than the NA-hydrolyzing activity (Table 1). Only limited NB-hydrolyzing activity was detected. Hence, butyrylesterases were only minor constituents of the corn-ester hydrolase complex. DFP at a concentration of 10^{-3} M slightly inhibited the activity of both NA- and NPOA-hydrolyzing enzymes (Table 2). The presence of A-ester hydrolases is consequently suggested. The extensive hydrolysis of NPOA, the slight effect of DFP, and the intermediate response to PCMB suggest that enzymes of the phenoxyester hydrolase type were possibly also present.

The separation of ester hydrolases in plant tissue into 7 to 9 distinct bands on the gels with NA as substrate is comparable with the situation in animal tissue. Up to 10 different ester hydrolases have been demonstrated in epididymal tissue of the mouse,[11] in a variety of adult mouse tissue,[12] and in adrenal medullary tissue of the rat.[13] Allen[14,15] separated about 20 bands which had ester hydrolase activity on starch gels from extracts of *Tetrahymena pyriformis.*

Classification of a complex array of enzymes such as the ester hydrolases presents a challenge to the investigator. The need for an extension of classification of enzymes beyond that based on substrate specificity alone was recognized by Market and Møller.[16] The term isozyme was suggested to resolve the physically distinct, multiple-molecular forms in which proteins with the same enzymatic specificity may exist. They considered that enzymes with broad substrate specificity should be grouped into families.[16] The members of the family would possess overlapping, but distinct patterns of substrate specificity.

In animals each tissue is considered to have its own characteristic pattern and proportion of isozymes, and the pattern and proportions change with the differentiation of the cells from embryonic stages to maturity.[16] In the unicellular *Tetrahymena*, Allen[15] postulated that each isozyme may have a different site of attachment within the cells.

The existence of isozymes among the plant ester hydrolases was not definitely established in this study; however, results of the electrophoretic studies suggest that they probably do exist.

The ester hydrolase situation in plant tissue is conceivably more complex than was indicated in the present study. Some of the ester hydrolases may not have been solubilized by the extraction techniques. The non-mobile activity at the origin in the gels might be traced to

[11] J. M. ALLEN and R. L. HUNTER, *J. Histochem. and Cytochem.* **8**, 50 (1960).
[12] C. L. MARKERT and R. L. HUNTER, *J. Histochem. and Cytochem.* **7**, 42 (1959).
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[15] S. L. ALLEN, *Ann. N. Y. Acad. Sci.* **94**, 753 (1961).
[16] C. L. MARKERT and F. MØLLER, *Proc. Nat. Acad. Sci. U.S.* **45**, 753 (1959).

ester hydrolases that were adsorbed onto particles.[17] The streaked areas in the gels adjacent to the origin could also be caused by enzymes that were not completely freed from particulate matter.

The contribution of the ester hydrolase activity of peptide hydrolases in the hydrolysis of substrates used in this study was not determined. Peptide peptidohydrolases such as trypsin, chymotrypsin, and papain are known to hydrolyze certain carboxyl esters. They catalyze hydrolysis by the same general mechanism as ester hydrolases and are inhibited by DFP and other ester hydrolase inhibitors.

If the status of ester hydrolases in plants and animals is similar, one could anticipate that individual members of the plant-ester hydrolase complex might (a) have different sites of attachment within the cells, (b) vary in different tissue, (c) differ with the stage of cellular differentiation, (d) vary with environmental conditions during growth, and (e) vary between species and varieties or strains within a given species.

EXPERIMENTAL

Preparation of Enzyme Extracts

Wheat seeds (*Triticum aestivum* L. "Anderson") were ground in a Wiley mill to pass a 40-mesh sieve. The resultant flour was defatted with petroleum ether. Enzyme extracts were prepared as described by Singer and Hofstee.[18] Acetone powders were prepared from the above-ground parts of 5- to 7-day-old greenhouse-grown cucumber (*Cucumis sativus* L. "Stono"), soybean [*Glycine max* (L.) Merr. "Lee"], and corn (*Zea mays* L. "GT 112 × CI. 21") seedlings. The acetone powders were extracted with 20 vols. of cold 0·05 M potassium phosphate buffer (pH 7·0) by continuous stirring in an icebath for 20 min. The resultant slurry was filtered through cheesecloth and centrifuged at 12,000 × *g* for 20 min in a Servall refrigerated centrifuge. The supernatant was used as the enzyme source in the substrate-specificity and inhibitor studies.

Substrates

NPOA and NB were synthesized as described by Gomori.[19] NA was obtained from the Dajac Laboratories and NC was supplied by H. A. Ramsey.

Assay Procedure

The hydrolysis of 2-naphthyl esters was determined by the method of Ramsey.[20] The 2-naphthyl esters were dissolved in 1,4-dioxane with Brij 35 (Atlas Powder Co.) as an emulsifier. Enzyme assays were performed at pH 7·0 (0·03 M potassium phosphate buffer), a temperature of 37°C, and a 20-min incubation period. The Brij 35 concentration in the reaction mixture was 0·83 per cent (w/v). Released 2-naphthol was coupled with a diazonium salt (4-sulfamoylbenzenediazonium chloride) to form an azo dye, the concentration of which was measured photometrically with a Klett-Summerson colorimeter (No. 42 filter).

Starch-gel electrophoresis

Partially hydrolyzed starch, prepared according to the method of Smithies,[21] was obtained from Connaught Medical Research Laboratories, University of Toronto, Toronto,

[17] R. L. HUNTER and M. S. BURSTONE, *J. Histochem. and Cytochem.* **8**, 58 (1960).
[18] T. P. SINGER and B. H. J. HOFSTEE, *Arch. Biochem.* **18**, 229 (1948).
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[21] O. SMITHIES, *Biochem. J.* **61**, 629 (1955).

Canada. The electrophoresis apparatus was constructed and the gel prepared as described by Smithies.[22] Electrophoresis was conducted with 0·025 M borate buffer (pH 8·6) at 3°C for 15 hr and a potential gradient of 8 V/cm. Measured aliquots of the enzyme preparations were applied to uniform-sized strips of filter paper. The paper strips were inserted in transverse slits cut in the gels. In each run 3 strips were inserted in the gels, ca. 2 cm apart. After electrophoresis the gels were sliced horizontally; hence, a single run provided 6 strips. The strips were incubated separately in a substrate-diazonium salt mixture described by Markert and Hunter.[12] The incubation mixture contained 100 ml water, 10 ml 0·2 M potassium phosphate buffer (pH 7·0), 1 ml of a 2-naphthyl ester stock solution (1 per cent ester in 1,4-dioxane), 2 ml Triton X-100 (Rohm & Haas Co.), and 100 mg Naphthanil Diazo Blue B (Dajac Laboratories). After a development period of 1hr the gels were washed in running tap water for several hours.

The enzyme preparations initially obtained were too dilute to be used directly in the electrophoretic studies. Consequently, enzymes in the extracts were precipitated by the addition of sufficient solid ammonium sulphate to produce 75 per cent saturation. The resultant precipitate was collected by centrifugation and resuspended in a small volume of 0·05 M potassium phosphate buffer pH 7·0. All the ester hydrolase activity in the crude extracts, as assayed with 2-naphthyl esters of acetic, butyric, and phenoxyacetic acid, could be accounted for in the precipitate.

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