Plant Physiol. (1980) 66, 731–734
0032-0889/80/66/0731/04$00.50/0

Characterization of α-Galactosidase from Cucumber Leaves[1]

Received for publication December 26, 1979 and in revised form June 9, 1980

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ABSTRACT

Two forms of α-galactosidase (α-D-galactoside galactohydrolase, E.C. 3.2.1.22) which differed in molecular weight were resolved from *Cucumis sativus* L. leaves. The enzymes were partially purified using ammonium sulfate fractionation, Sephadex gel filtration, and diethylaminoethyl-Sephadex chromatography. The molecular weights of the two forms, by gel filtration, were 50,000 and 25,000. The 50,000-dalton form comprised approximately 84% of the total α-galactosidase activity in crude extracts from mature leaves and was purified 132-fold. The partially purified 25,000-molecular weight form rapidly lost activity unless stabilized with 0.2% albumin and accounted for 16% of the total α-galactosidase activity in the crude extract. The smaller molecular weight form was not found in older leaves.

The two forms were similar in several ways including their pH optima which were 5.2 and 5.5 for the 50,000- and 25,000-dalton form, respectively, and activation energies, which were 15.4 and 18.9 kilocalories per mole for the larger and smaller forms. Both enzymes were inhibited by galactose as well as by excess concentrations of *p*-nitrophenyl-α-D-galactoside substrate. K_m values with this substrate and with raffinose and melibiose were different for each substrate, but similar for both forms of the enzyme. With stachyose, K_m values were 10 and 30 millimolar for the 50,000- and 25,000-molecular weight forms, respectively.

The present study involved the resolution, partial purification, and characterization of two forms of α-galactosidase in cucumber leaves as a step in understanding the role of α-galactosidase in the breakdown of the transport sugars, stachyose and raffinose (17). Several properties of the two forms were compared, including their mol wt, substrate and inhibitor specificities, activation energies, and their occurrence as a function of leaf age.

α-Galactosidase (α-D-galactoside galactohydrolase, E.C. 3.2.1.22) has also been studied in squash (15, 16), another cucurbit that transports stachyose. Thomas and Webb (15, 16), using DEAE-Sephadex[4] ion exchange chromatography, separated three isozymic forms from squash leaves and studied their distribution in the plant. Substrate specificity studies were conducted with raffinose, but not with stachyose or melibiose (15). An α-galactosidase in spinach leaves was studied by Gatt and Baker (5). No estimates of mol wt were given in either of these studies.

The majority of work on plant α-galactosidases has focused on seed tissues since seeds contain large amounts of sugars of the raffinose family which are utilized as readily available energy sources during germination. Many seeds have been found to contain a larger and a smaller mol wt form of α-galactosidase. Larger forms ranged in weight from 125,000 to 209,000 daltons, with smaller forms weighing from 23,000 to 57,000 daltons (3, 6). Dey and Pridham (2, 13), for example, characterized a 38,000- and a 209,000-mol wt form from *Vicia faba* seeds. Other seeds have only one form, either in the larger or in the smaller weight range (3, 6).

MATERIALS AND METHODS

Plant Material. Cucumber plants (*Cucumis sativus* L. cv. Chipper) were grown in a greenhouse. Mature, fully expanded leaves at the seventh node from the growing tip of 40- to 45-day-old plants were used for enzyme extractions.

Enzyme Extraction. Crude extracts were prepared by homogenizing 25 g of cucumber leaves with 80 ml of 0.1 M Na-acetate buffer (pH 5.2) for 3 min in a VirTis homogenizer. The homogenate was centrifuged at 27,000*g* for 15 min, and the supernatant was retained and filtered through cheesecloth.

Assays for α-Galactosidase. α-Galactosidase activity was monitored routinely using 1 mM *p*-nitrophenyl-α-D-galactoside as substrate at pH 4.6 in 0.1 M Na-acetate buffer and at 30 C, as described by Pharr *et al.* (11). One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 μmol *p*-nitrophenol/min under the above conditions.

When stachyose, raffinose, and melibiose were used as substrates, α-galactosidase activity was measured by a fixed time assay scheme of Gatt and Baker (5), using β-galactose dehydrogenase to detect galactose produced. Reaction mixtures contained α-galactosidase preparation and 16 mM substrate in 0.16 M Na-acetate buffer (pH 5.0), and were incubated at 30 C for varying times up to 4 h depending upon the substrate used. A unit of α-galactosidase activity was expressed in terms of 1 μmol galactose released/min.

Assays for Contaminating Enzymes. The presence of invertase in final enzyme preparations was monitored by observing the increase in reducing groups produced by hydrolysis of sucrose using Nelson's reducing sugar test (10).

The assay scheme using β-galactose dehydrogenase for galactose detection (5) was modified to test for alkaline phosphatase and for acid phosphatase contamination. In the case of alkaline phosphatase, a reaction mixture containing an α-galactosidase preparation and 6.0 mM galactose-1-phosphate in 0.1 M Na-acetate (pH 5.0) was brought immediately to pH 8.6 by the addition of 0.5 ml Tris-GHS-NAD^+ solution. The galactose detection procedure as outlined by Gatt and Baker (5) was then followed. The β-galactose dehydrogenase preparation used was tested and found free of contaminating alkaline phosphatase activity when the assay was run minus the α-galactosidase enzyme component.

[1] This work was supported in part by funds from Pickle Packers International and United States Department of Agriculture, Science and Education Administration cooperative agreement No. 58-7B30-9-140 and is paper 6262 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, North Carolina.

[2] This research was done as a partial fulfillment of the requirements for the PhD degree at North Carolina State University.

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[4] The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Experiment Station of products named nor criticism of similar ones not mentioned.

In the test for acid phosphatase activity, 6.0 mM galactose-1-phosphate was used as substrate in 0.1 M Na-acetate buffer (pH 5.0). Because we had already determined that no alkaline phosphatase was present, the procedure of Gatt and Baker (5) was followed and any detectable activity was attributed to the presence of acid phosphatase.

Protein Determination. Protein was monitored routinely by *A* at 280 nm, assuming 1 mg protein/ml gives an *A* of 1.0.

Enzyme Resolution and Partial Purification. Partial purifications of a 50,000- and a 25,000-mol wt form of α-galactosidase were achieved by ammonium sulfate fractionation and by gel filtration with Sephadex G-75 in two successive runs. The 50,000-mol wt sample was purified further by ion exchange chromatography on a DEAE-Sephadex A-50 column. All extraction and purification steps were carried out at 4 C.

A 25-g sample of cucumber leaves was homogenized with 80 ml of 0.1 M Na-acetate buffer (pH 5.2) for 3 min. The homogenate was centrifuged at 27,000*g* for 15 min, and the supernatant was retained. The supernatant was brought to 30% saturation with ammonium sulfate and centrifuged. The supernatant was retained and brought to 60% saturation by further addition of ammonium sulfate. After centrifuging at 27,000*g* for 15 min, the supernatant was discarded, and the pellet was suspended in about 10 ml of 50 mM Na-phosphate buffer (pH 7.0) containing 0.1% BSA. It was necessary to add BSA to this column sample and to the subsequent column elution buffers to stabilize the 25,000-dalton form, which otherwise lost 60% of its activity within 3 h after eluting from a column. The BSA prevented estimation of the degree of purification of the 25,000-dalton enzyme, but not of the 50,000-dalton enzyme since the BSA did not coelute with the larger enzyme from the ion exchange column.

The dissolved pellet was applied to the first Sephadex G-75 column (2.5 × 75 cm), which had previously been equilibrated with 50 mM Na-phosphate buffer (pH 7.0) containing 0.2% BSA. Elution was upflow with the same buffer at a flow rate of 14 ml/h, and 4-ml fractions were collected. The fractions comprising the major activity peak between 164 and 216 ml were pooled for further purification of the 50,000-mol wt form. Fractions comprising the minor peak between 220 and 252 ml were pooled for further purification of the 25,000-mol wt form (data not shown). Each pooled fraction was subjected independently to a second pass through the same Sephadex G-75 column to further resolve the two forms. The final 25,000-mol wt form product consisted of the pooled fractions that eluted between 216 and 256 ml from the second G-75 column (Fig. 3B).

The 50,000-mol wt enzyme product from the second, but separate, Sephadex G-75 run consisted of the pooled fractions between 174 and 220 ml. This sample was treated further by diluting it to 265 ml with distilled H_2O and applying it to a 95-ml DEAE-Sephadex A-50 column (2.5 × 19 cm), which had been equilibrated with 10 mM Na-phosphate (pH 7.0). Two bed volumes of buffer, 186 ml, were passed through the column to ensure that all of the α-galactosidase activity had adherred, before eluting with a linear Na-phosphate gradient, 460 ml ranging from 10 to 100 mM Na-phosphate (pH 7.0). The α-galactosidase activity peak eluted at 56 mM.

RESULTS AND DISCUSSION

Occurrence of Two Forms of α-Galactosidase as a Function of Leaf Age. To determine the effect of leaf age on the presence of different mol wt forms of α-galactosidase, the 30 to 60% ammonium sulfate fractions from cucumber leaves of three different ages were passed through a Sephadex G-100 column (2.5 × 55 cm). Youngest leaves, mature leaves of intermediate age, and oldest leaves were from the second, seventh, and fifteenth nodes for the growing tip, respectively, of 40-day-old plants. Elution profiles for young leaves and for mature leaves showed a principal activity peak reflecting a larger form, followed by a smaller peak reflecting a lower mol wt form (Fig. 1, A and B). The profile for oldest leaves lacked the smaller peak (Fig. 1C).

Absence of the lower mol wt form in older leaves might indicate that cells had ceased to synthesize this form of the enzyme, or that it is less stable in older leaves. Total α-galactosidase activity in crude extracts, expressed as μmol of *p*-nitrophenol produced/min · g of leaves ranged from 0.22 to 0.32 for youngest and intermediate age leaves, and 0.35 to 0.52 for oldest leaves, based on three independent leaf samples. In terms of milliunits/mg protein, determined by the method of Lowry *et al.* (8), the average of three readings was 22.2 ± 0.2, 23.0 ± 5.0, and 29.0 ± 6.6 for young, intermediate, and oldest leaves, respectively.

To examine the possibility that the smaller form might have been an artifact of ammonium sulfate fractionation, a crude extract which had not been treated with ammonium sulfate was passed through a Sephadex G-75 column. The elution profile (Fig. 3A) was the same with respect to the positions and proportion of the major and minor peaks as that obtained with extracts treated with ammonium sulfate (Fig. 1). The presence of 0.2% BSA in extract and elution buffers had no effect on the elution profile. Thus, BSA was not needed to stabilize the smaller enzyme form at this earlier point in the purification, although later it was necessary. The larger mol wt form comprised an estimated 83% of total α-galactosidase activity measured, whereas the smaller form made up 15% (Fig. 3A). The remaining 2% activity measured was in a very small shoulder eluting ahead of the larger mol wt form.

Mol Wt of the α-Galactosidase Forms. Mol wt of the two forms of α-galactosidase were estimated from a plot of the log mol wt of

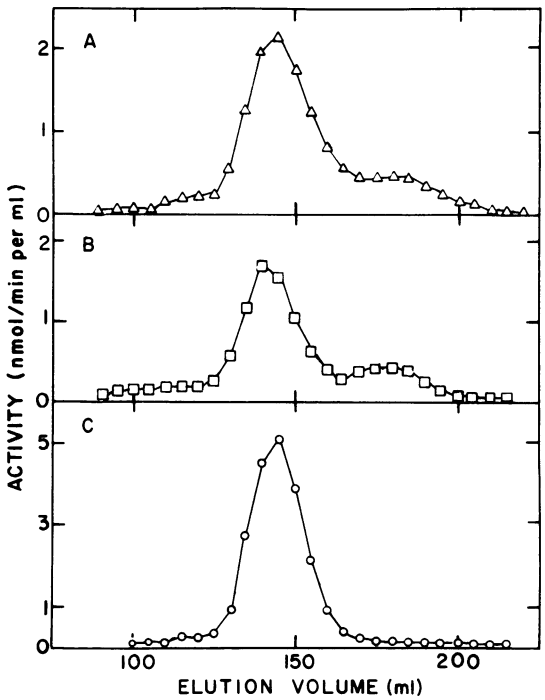

FIG. 1. Elution profiles from a Sephadex G-100 column for ammonium sulfate fractions from three different age leaves. A, youngest leaves were from the second node from the growing tip and had a fresh weight of 0.75 g/leaf. B, mature leaves of intermediate age were from the seventh node from the growing tip and weighed 3 to 4 g each. C, oldest leaves were from the fifteenth node from the growing tip and weighed 3 to 4 g each. Oldest leaves were chlorotic along leaf edges. Samples were crude extracts that had been precipitated between 30 and 60% ammonium sulfate. Two-ml samples, each the equivalent from 0.38 g of leaves, were applied upflow to a Sephadex G-100 column (2.5 × 55 cm) that was equilibrated and eluted with 50 mM Na-phosphate (pH 7.0).

marker proteins of varying mol wt *versus* their peak elution volume from a Sephadex G-100 column (2.5 × 55 cm) (Fig. 2). The mol wt of the smaller, less abundant form of α-galactosidase was 25,000, whereas the estimated mol wt for the larger, predominant form was 50,000.

Enzyme Resolution and Partial Purification. The partial purification procedure successfully resolved the two forms from each other and from certain enzymes which would interfere in kinetic studies. Table I gives the results from the five-step procedure for the 50,000-mol wt form of α-galactosidase. The 25,000-mol wt form was prepared by using the same first four purification steps. Over-all recovery of both forms was 14% and a 132-fold purification of the 50,000-dalton form was achieved. No estimation of the purity of the 25,000-dalton form was possible due to the presence of the stabilizing BSA in the column elution buffers. The final 25,000-mol wt form enzyme product consisted of 44 ml, containing a total enzyme activity of 0.08 units.

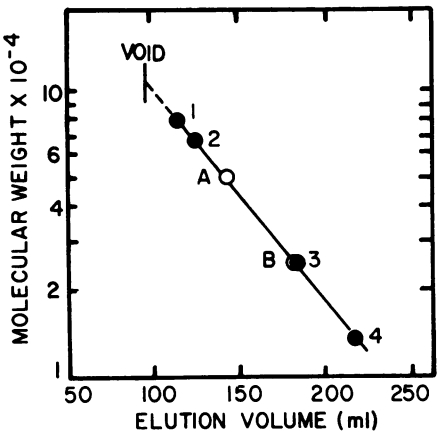

FIG. 2. Calibration curve from which the mol wt of two forms of α-galactosidase were obtained. Samples of partially purified forms of α-galactosidase A and B, as well as: 1, alkaline phosphatase from *E. coli* type III-R (mol wt 80,000); 2, BSA (mol wt 67,000), 3, chymotrypsinogen from bovine pancreas (mol wt 25,000); and 4, ribonuclease A from beef pancreas (mol wt 13,700) were applied upflow to a Sephadex G-100 column (2.5 x 55 cm). All samples were 2 ml in volume, except alkaline phosphatase which was applied in 1 ml. Five-ml fractions were collected. A line was fitted to the calibration proteins by regression analysis. Point A was the peak elution volume (145 ml) of one form of α-galactosidase and corresponded to a mol wt of 50,000. Point B was the peak elution volume (185 ml) of the other form of α-galactosidase and corresponded to a mol wt of 25,000.

Table I. *Preparation of 50,000-mol wt Form of α-Galactosidase*

Step	Volume	Total Protein by A_{280}	Total Enzyme Activity	Specific Activity	Over-all Recovery	Purification
(From 25 g of leaves)	*ml*	*mg*	*μmol/min*	*μmol/min·mg × 10³*	*%*	*fold*
Crude extract	85	2450	4.27	1.74		1.0
Ammonium sulfate 30–60%	9	360	2.75	7.64	64	4.4
First G-75	30	[a]	2.33	[a]	55	[a]
Second G-75	53	[a]	1.42	[a]	33	[a]
DEAE A-50	70	2.52	0.58	230	14	132[b]

[a] Measurements at these points were not made due to presence of BSA in column elution buffers.

[b] Approximately 83% of activity in the crude extract was 50,000-mol wt enzyme. Based on this value, the over-all purification was 161 and over-all recovery was 17%.

The 50,000-mol wt sample was free of contamination by the 25,000-mol wt form. To check on contamination, a sample of pooled DEAE-purified 50,000-mol wt enzyme was passed through a G-75 column (2.5 × 73 cm). There was no activity eluting at 230 ml, the point at which 25,000-mol wt form enzyme would peak (Fig. 3C).

Both enzyme preparations were free of the contaminating enzyme invertase. The 50,000-mol wt form sample was also tested for alkaline phosphatase and acid phosphatase contamination. No alkaline phosphatase was detected and the amount of acid phosphatase contamination was too small to interfere with inhibitor studies. The 50,000-mol wt enzyme was stable for at least 7.5 months when stored at 0 C either in the presence of 1 mg/ml BSA or in its absence. The 25,000 form was stable for at least 1 month in the presence of 1 mg/ml BSA.

General Properties. Optimum pH values for the 25,000- and 50,000-mol wt forms were 5.5 and 5.2, respectively. Characterization studies of the two forms were carried out in the Na-acetate buffer systems at the pH optima. The 50,000-mol wt enzyme exhibited a broader pH optimum range, from a pH of 4.0 to 5.6, than did the 25,000-dalton form when tested with a Na-citrate buffer system and a Na-acetate combined with a Na-phosphate buffer system (data not shown).

Dey and Pridham (1) reported significant differences in the activation energies for two mol wt forms of α-galactosidase in *Vicia faba* seeds: 27.2 and 15.3 kcal/mol for a smaller and larger form, respectively. By comparison, activation energies for the hydrolysis of *p*-nitrophenyl-α-D-galactoside by the cucumber leaf α-galactosidases, as calculated from Arrhenius plots at temperatures from 5 to 30 C, were similar: 18.9 and 15.4 kcal/mol for the 25,000- and 50,000-mol wt forms, respectively (data not shown).

Inhibitor Studies. There was no inhibition of activity of the 132-fold purified 50,000-mol wt α-galactosidase by sucrose, galactose-1-phosphate, UDP-galactose, or UDP-glucose at concentrations up to 20 mM, when assayed with 6 mM raffinose. The 25,000-mol wt form was not tested because of insufficient enzyme preparation. The above compounds are of interest since they are likely intermediates in stachyose catabolism. The presence or absence of a contaminating epimerase and phosphodiesterase was not established, and these could conceivably have altered the concentrations of UDP-galactose and UDP-glucose used to determine inhibition. Given these reservations, there would appear to be no regulation of the 50,000-mol wt α-galactosidase activity through feedback inhibition by the compounds tested.

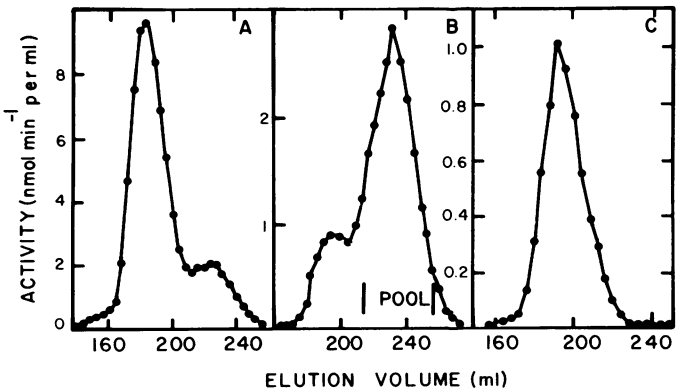

FIG. 3. Elution profiles from Sephadex G-75 columns. A, profile for a crude extract leaf sample; B, profile from the second G-75 step in the preparation of 25,000-mol wt form of α-galactosidase showing fractions pooled for the final 25,000-dalton sample; C, profile for DEAE-purified 50,000-mol wt sample of α-galactosidase verifying freedom from contamination by 25,000-mol wt form. All columns measured 2.5 × 73 cm and were equilibrated and eluted upflow with 50 mM Na-phosphate (pH 7.0).

Galactose has been shown to be a powerful and competitive inhibitor of α-galactosidases (2, 4, 14). Hyperbolic curves for the two forms of cucumber leaf α-galactosidase were almost superimposable and showed 50% inhibition at 2 mM galactose, when activity was assayed at a saturating level (1 mM) of *p*-nitrophenyl-α-D-galactoside. Purely competitive inhibition with galactose was verified for the 50,000-mol wt form, but was assumed for the 25,000-mol wt form because of insufficient enzyme. Purely competitive inhibitor constants (K_i values) for the two forms were 0.65 and 0.45 mM for the 25,000- and 50,000-dalton forms, respectively, as estimated using plots of $\frac{1}{v}$ *versus* [I] with additional data for the K_m of each enzyme (data not shown).

Substrate Specificity. Of 10 nitrophenyl glycosidases tested as substrates for the 50,000-mol wt α-galactosidase, only the *p*-nitrophenyl-α-D-galactoside was hydrolyzed. The compounds included *p*-nitrophenyl-α-glucoside, -β-glucoside, -α-galactoside, -β-galactoside, -α-mannoside, -β-mannoside, -α-xyloside, -β-xyloside, -β-fucoside, and *o*-nitrophenyl-β-glucoside. This indicated that the α-galactosidase was highly specific for both the galactose and the α-configuration, and that the 50,000-mol wt preparation was free from several other glycosidases.

Hydrolysis of *p*-nitrophenyl-α-D-galactoside, with the 25,000 and with the 50,000-mol wt form, did not follow Michaelis kinetics at high substrate concentrations and showed inhibition at concentrations greater than 2 mM. K_m values were 0.32 mM for the 25,000-mol wt form and 0.22 mM for the 50,000-mol wt form (Table II).

Substrate studies with stachyose, raffinose, and melibiose could be carried out, since both enzyme preparations lacked the contaminating enzyme invertase. With stachyose, raffinose, and melibiose as substrates, plots of (s)/v against (s) resulted in straight lines with both forms of the enzyme. No inhibition in the presence of excess substrate was observed. With raffinose as substrate, K_m values for the 50,000- and 25,000-dalton forms were 6.5 mM and 5.0 mM, respectively (Table II). By comparison, three forms of α-galactosidase from mature squash leaves (15) had K_m values of 11.1, 12.5, and 5.6 mM. K_m values for the two forms of α-galactosidase from cucumber leaves were different when stachyose was used as substrate: 10 mM for the 50,000-mol wt form and 30 mM for the 25,000-mol wt form (Table II). The 50,000-mol wt form hydrolyzed raffinose at 2.6 times the rate of stachyose and 7.7 times the rate of melibiose whereas the 25,000-mol wt form hydrolyzed raffinose at 2.6 times the rate of stachyose and 5.6 times the rate of melibiose.

Table II. *Substrate Specificity of 50,000-mol wt and 25,000-mol wt Form of α-Galactosidase*

Substrate	50,000-mol wt Form		25,000-mol wt Form	
	K_m	V_{max}	K_m	V_{max}
	mM	μmol product/min·ml enzyme	mM	μmol product/min·ml enzyme
p-Nitrophenyl-α-D-galactoside	0.22	9.77×10^{-3}	0.32	3.20×10^{-3}
Melibiose (Gal-Glu)	3.0	0.83×10^{-3}	3.5	0.38×10^{-3}[a]
Raffinose (Gal-Glu-Fruc)	6.5	6.57×10^{-3}	5.0	2.08×10^{-3}[a]
Stachyose (Gal-Gal-Glu-Fruc)	10.0	2.52×10^{-3}	30.0	0.82×10^{-3}[a]

[a] The 25,000-mol wt enzyme sample was concentrated to approximately ⅔ of the original volume to obtain V_{max} values; therefore, it is not possible to compare these three values for V_{max} with the V_{max} value obtained when *p*-nitrophenyl-α-D-galactoside was used as substrate.

The α-galactosidases described here were identified primarily by their ability to hydrolyze *p*-nitrophenyl-α-D-galactoside. At least some forms of plant glycosidases have been reported which show little or no activity toward *p*-nitrophenyl-β-glucoside and yet catalyze the hydrolysis of β-glucosides found in the plant (7, 9, 12). If an α-galactosidase of this type were present in cucumber leaves, it may not have been identified in the present study.

The 25,000- and 50,000-mol wt forms of α-galactosidase from cucumber leaves show many similar properties, including pH optima, activation energies, and inhibition and substrate kinetics. Considering these similarities and the mol wt of the two forms, it seems possible that the less stable 25,000-dalton form is a subunit or a precursor to the more stable and predominant 50,000-dalton form.

Acknowledgment—We wish to thank Dr. A. R. Main of the Department of Biochemistry at North Carolina State University for helpful discussions of kinetic aspects of this work.

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