Plant Physiol. (1971) 47, 114–118

Assay of Adenosine 5′-Triphosphate Sulfurylase by Pyrophosphate Exchange

Received for publication July 27, 1970

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ABSTRACT

The sulfate-dependent pyrophosphate exchange reaction has been re-examined and confirmed. Standard assay conditions for measuring ATP sulfurylase by sulfate-dependent pyrophosphate exchange are described and some properties of the enzyme (measured in crude dialyzed extracts) are reported. This method has many advantages over the well established molybdate method.

Marcus (11), in a study of the aminoacyl-tRNA synthetases of spinach leaf tissue, reported that when magnesium sulfate was used in lieu of magnesium chloride as the source of Mg^{2+}, very high endogenous pyrophosphate exchange activity was obtained. Marcus ascribed this activity to sulfate activation (ATP: sulfate adenyltransferase, E.C.2.7.7.4; trivial name, ATP sulfurylase).

Subsequently, Asahi (5) reported the incorporation of ^{35}S-sulfate into APS[1] in isolated chloroplasts and since then ATP sulfurylase has been examined in plants by several groups. The most definitive work on the enzymology of ATP sulfurylase is that of Ellis (7) who has developed a chromatographic technique for measuring the APS formed; this method is reliable but tedious and unsatisfactory for use in conjunction with enzyme purification. Adams and co-workers (1, 2) on the other hand have developed the molybdate method of Wilson and Bandurski (13) to measure ATP sulfurylase in several plants. The basis of this method is that molybdate is used as an analogue of sulfate, forming the unstable adenosylphosphomolybdate and pyrophosphate. Molybdate is used because the equilibrium favors the formation of product more than if sulfate is used as substrate (13), thus producing a more favorable yield of product. The pyrophosphate produced is hydrolyzed by endogenous pyrophosphatase present in the extract, and the amount of phosphate produced is then measured and used as an index of enzyme activity. The molybdate method has several serious disadvantages: (*a*) the assay is conducted with the wrong substrate, (*b*) ATPase causes a very high endogenous formation of phosphate, (*c*) the hydrolysis of pyrophosphate must be complete; it is essential to assay the pyrophosphatase present in the extract before conducting the ATP sulfurylase assay to establish that there is sufficient pyrophosphatase activity to accomplish complete hydrolysis; otherwise, additional pyrophosphatase must be added. Indeed the only evidence for inferring that the molybdate method is valid for assaying ATP sulfurylase in plant extracts is that the amount of molybdate-dependent phosphate produced is inhibited by sulfate (1).

[1] Abbreviation: APS: adenosyl-5′-phosphosulfate.

We have re-examined the sulfate-dependent pyrophosphate exchange reaction reported by Marcus (11) and confirmed his initial observation. We have found the pyrophosphate exchange technique superior to any of the other methods published to date; the method is extremely rapid, no prior assay of pyrophosphatase is required, sulfate is used as substrate, and the method is much more sensitive than the established molybdate method. Some properties of the ATP sulfurylase of spinach leaf tissue using the pyrophosphate exchange technique are reported.

MATERIALS AND METHODS

Chemicals. ATP (disodium salt) was supplied by C. F. Boerhinger und Soehne, G. m. b. H, Mannheim, Germany, and solutions were adjusted to pH 7.5 with KOH. ^{32}P-Orthophosphate was supplied by the Australian Atomic Energy Commission, Lucas Heights, Sydney, N. S. W., Australia, and converted into ^{32}P-pyrophosphate by pyrolysis (10). ^{32}P-Pyrophosphate was diluted with unlabeled pyrophosphate to adjust the specific radioactivity to approximately 0.25 $\mu c/\mu mole$.

Spinach (*Spinacia oleracea*) was grown in a glass house and used immediately after harvest. The ribs of the leaves were discarded, and the leaf tissue was extracted in a pestle and mortar using 2 ml of medium 1 (100 mM tris-HCl buffer, pH 7.5, containing 50 mM $MgCl_2$) per gram fresh weight. The extract was passed through muslin and then centrifuged at 500*g* for 5 min and the supernatant solution was recentrifuged at 30,000*g* for 60 min. The supernatant solution was dialyzed against 70 volumes of medium 2 (20 mM tris-HCl, pH 7.5, containing 50 mM $MgCl_2$) for 20 hr with two changes. The dialyzed solution was diluted 4-fold in medium 2 and was used as the source of crude dialyzed enzyme. Addition of NaCl (0.35 M) or potassium thioglycolate (1 to 25 mM) to medium 1 did not affect the amount of enzyme activity extracted from the tissue. All operations were carried out at 1 C.

Assay of ATP Sulfurylase. Standard incubation mixtures contained 100 μmoles of tris-HCl, pH 7.5, 2 μmoles of Na_2K_2ATP, 2 μmoles of ^{32}P-pyrophosphate, 10 μmoles of NaF, 25 μmoles of $MgCl_2$, 40 μmoles of K_2SO_4, and 0.5 ml of diluted enzyme in a final volume of 1 ml; the values for tris and $MgCl_2$ are corrected for the amounts introduced with the enzyme sample. K_2SO_4 was omitted from control incubations. Assays were conducted at 35 C and were terminated after 15 min by the addition of 2 ml (7.5%, w/v) of trichloroacetic acid. ^{32}P-ATP was adsorbed onto charcoal and separated from ^{32}P-pyrophosphate as described by Anderson (4), and the radioactivity of the ATP adsorbed onto the charcoal was counted. Enzyme activity is expressed as the difference in the ATP-pyrophosphate exchange rates (in nmoles/min; *i.e.*, ATP sulfurylase units) determined for assay mixtures with and without added sulfate. Specific ATP sulfurylase activity is defined as ATP sulfurylase units/mg of protein. Rate of exchange was calculated by the method of Davie *et al.* (6), but usually the enzyme was diluted prior to assay to reduce the exchange to less than 5% so

that the amount of pyrophosphate exchanged was proportional to the amount of label in the ATP. When dialyzed crude extract was replaced by medium 2 in the standard assay, no radioactivity was adsorbed by the charcoal. All results are the means of duplicate determinations.

Chromatography of the Radioactive Product Adsorbed onto Charcoal. Radioactive material was eluted from charcoal with 0.1 M NH_3 in 50% (v/v) ethanol and the eluate was evaporated to dryness. The residue was dissolved in water, and samples were subjected to descending chromatography on acid-washed Whatman No. 3 filter paper using the following solvents: (*a*) propyl acetate–formic acid–water (11:5:3, v/v, 16 hr); (*b*) *n*-propanol–NH_3–water (6:3:1, v/v, 50 hr); (*c*) isobutyric acid–NH_3–water (66:1:33, v/v, 16 hr).

Determination of Protein. Protein was precipitated with trichloroacetic acid (10%, w/v), washed twice with acetone, solubilized in 2 M NaOH at 50 C for 2 hr, and protein determined by the method of Ellman (8) with crystalline bovine serum albumin as standard.

Assay of ATPase and Pyrophosphate Activities. ATPase and pyrophosphatase activities were measured in diluted dialyzed extracts as used for the ATP sulfurylase assay. Incubation mixtures contained 0.5 ml of extract, 2 μmoles of sodium pyrophosphate or Na_2K_2ATP, 100 μmoles of tris-HCl, and 25 μmoles of $MgCl_2$; the values for $MgCl_2$ and tris include the amounts introduced with the enzyme aliquot. Sometimes NaF (10 μmoles) was included in the assay but this is specified for each experiment. Assays were conducted at 35 C and terminated after 15 min by the addition of 5 ml (12%, v/v) of perchloric acid. The terminated reaction mixtures were analyzed for phosphate (3) and corrected for phosphate present in reaction mixtures terminated at zero time. Since ATPase and pyrophosphatase activities were measured in relation to the activity of ATP sulfurylase, the activities are expressed as micromoles P_i released per milliliter incubating mixture per 15 min. All results are means of duplicate determinations.

RESULTS AND DISCUSSION

Undialyzed extracts were an unsatisfactory source of enzyme; the sulfate dependent pyrophosphate exchange of undialyzed extracts was only 15% greater than the endogenous pyrophosphate exchange. Dialysis decreased endogenous activity by approximately 90%; using dialyzed extracts under the standard assay conditions, a 6- to 8-fold stimulation of pyrophosphate exchange was observed on addition of sulfate (Fig. 1) and sulfate-dependent pyrophosphate exchange was a linear function of the amount of enzyme added. Figure 1 also illustrates the sensitivity of the pyrophosphate exchange assay. The rates of sulfate-dependent pyrophosphate exchange at 25, 30, and 35 C were constant for at least 20 min and the rates increased with temperature (Fig. 2). The rates at 40 and 45 C were initially higher than at 35 C but the rates at 40 and 45 C decreased after 12 and 8 min, respectively, presumably due to thermal denaturation of the enzyme. Therefore, 15 min at 35 C was selected for the standard assay. Preincubation of the dialyzed crude extract at 80 C for 10 min totally destroyed both endogenous and sulfate-dependent pyrophosphate exchange. When tris-maleic acid-KOH buffer (9) was used, maximum ATP sulfurylase activity was observed over a wide pH range but activity decreased sharply at pH values less than 5.5 (Fig. 3).

Magnesium sulfate was an unsatisfactory source of sulfate because this substrate does not permit separation of the sulfate- and Mg^{2+}-dependent pyrophosphate exchange reactions. A concentration of 20 to 40 mM magnesium chloride was necessary to saturate the Mg^{2+}-dependent activation of endogenous substrates; endogenous substrates probably include amino acids and

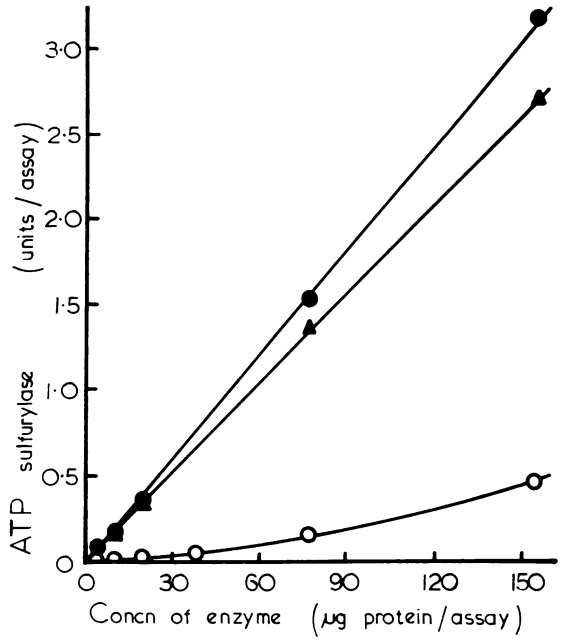


FIG. 1. Effect of enzyme concentration upon sulfate-dependent ATP-PP_i exchange activity (▲) under conditions of the standard assay. Activities with (●) and without (○) 40 mM K_2SO_4 are also included. A concentration of 0.1 mg protein/assay represents the protein extracted from 40 mg fresh weight of leaf tissue.

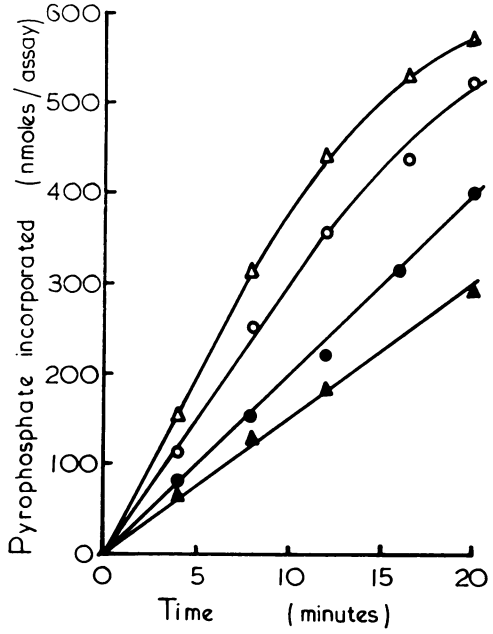


FIG. 2. Effect of time upon sulfate-dependent ATP-PP_i exchange activities at 25 C (▲), 35 C (●), 40 C (○), and 45 C (△).

amides (activated by aminoacyl-tRNA synthetases), fatty acids (activated by fatty acid thiokinases) as well as endogenous sulfate. A concentration of 20 to 40 mM magnesium chloride was also sufficient to saturate sulfate-dependent pyrophosphate exchange. Therefore, magnesium chloride was always used as the source of Mg^{2+} and potassium sulfate as the source of sulfate. ATP sulfurylase of spinach in dialyzed crude extracts has low affinity for sulfate when assayed by pyrophosphate exchange at a constant level of Mg^{2+} (Fig. 4); it is not possible to cite a K*m* value until

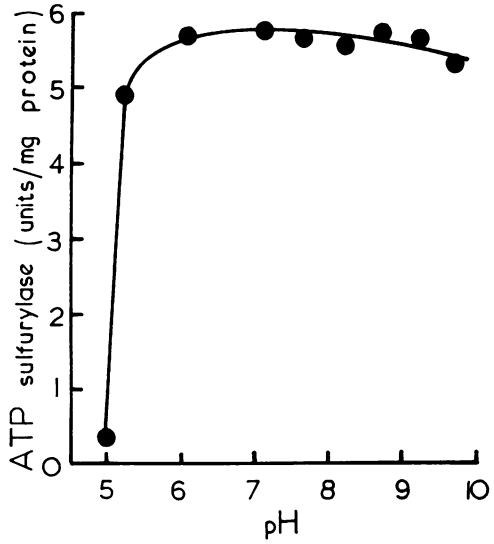


FIG. 3. Effect of pH upon ATP sulfurylase activity using tris-maleic acid-KOH buffer.

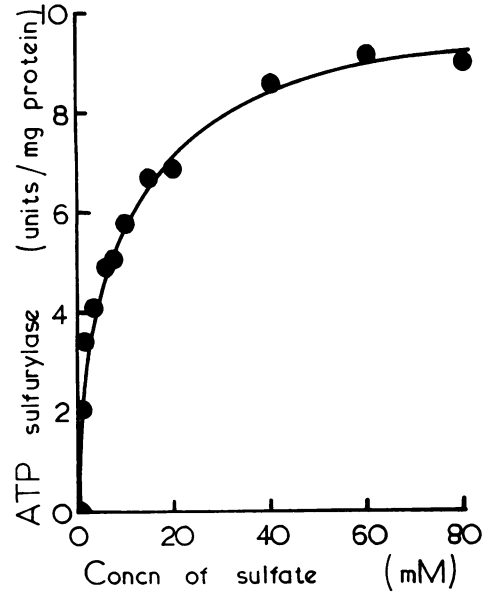
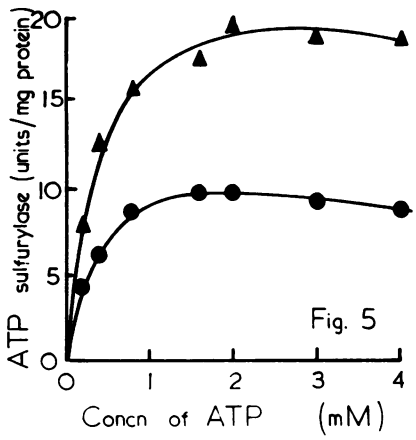


FIG. 4. Effect of concentration of K_2SO_4 upon ATP sulfurylase activity. ATP-PP_i exchange activity in the absence of K_2SO_4 was 1.01 units/mg protein.

the enzyme has been purified but the concentration of sulfate required to produce half maximum activity was approximately 2 to 8 mM. Similarly, concentrations of ATP and pyrophosphate required to produce half maximal activities were approximately 0.3 mM and 0.1 mM, respectively (Figs. 5 and 6).

Most of the radioactivity (91%) adsorbed by charcoal in standard assays with and without sulfate was eluted with 0.1 M NH_3 in 50% (v/v) ethanol. Chromatography of the eluates in solvent I demonstrated that the sulfate-dependent radioactivity adsorbed to charcoal was not pyrophosphate but ran with R_F similar to ATP (Fig. 7A–C). When the eluate derived from the assay containing sulfate was chromatographed in solvent III, all the radioactivity was cochromatographed with ATP (Fig. 7D) and no ^{32}P-ADP was detected. The radioactive product (obtained when the charcoal eluate was run in solvent I (Fig. 7A)) was eluted and rerun in solvents II and III, and the radioactive product was confirmed as ^{32}P-ATP.



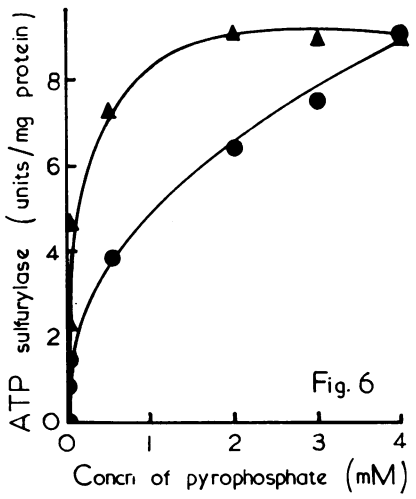


FIG. 5. Effect of concentration of ATP upon sulfate-dependent ATP-PP_i exchange activity with (▲) and without (●) 10 mM sodium fluoride.

FIG. 6. Effect of concentration of ^{32}P-sodium pyrophosphate (of constant specific radioactivity) upon sulfate-dependent ATP-PP_i exchange activity with (▲) and without (●) 10 mM sodium fluoride.

Sodium fluoride usually stimulated both endogenous and sulfate-dependent pyrophosphate exchange of dialyzed crude extracts approximately 2-fold (Fig. 5) and therefore 10 mM sodium fluoride was included in the standard assay. Fluoride has been included in assays of other pyrophosphate exchange reactions in plants (11, 12) to inhibit ATPase though fluoride also inhibited prolyl-tRNA synthetase of pea seeds (12). However, the data in Figure 5 shows that the enhancement of sulfate-dependent pyrophosphate exchange by fluoride cannot be obviated by increasing the concentration of ATP as might be expected if the action of fluoride was specifically to inhibit ATPase activity. ATPase activity in the dialyzed crude extract was low. Fluoride does inhibit ATPase (Table I), however, but assuming that the phosphate produced by the action of ATPase represents the amount of ATP hydrolyzed to ADP, then the amount of ATP hydrolyzed in the absence of fluoride is not sufficient in itself to account for the fluoride stimulation of ATP sulfurylase (Fig. 5).

The more important effect of fluoride in the ATP sulfurylase assay seems to be the inhibition of pyrophosphatase. Spinach leaf tissue contains a powerful pyrophosphatase. In one experiment using 2 μmoles of pyrophosphate as substrate, 1.88 μmoles of pyrophosphate was hydrolyzed to orthophosphate after 15 min; 10 mM fluoride almost completely inhibited pyrophosphatase (Table I). The hydrolysis of pyrophosphate in the absence of

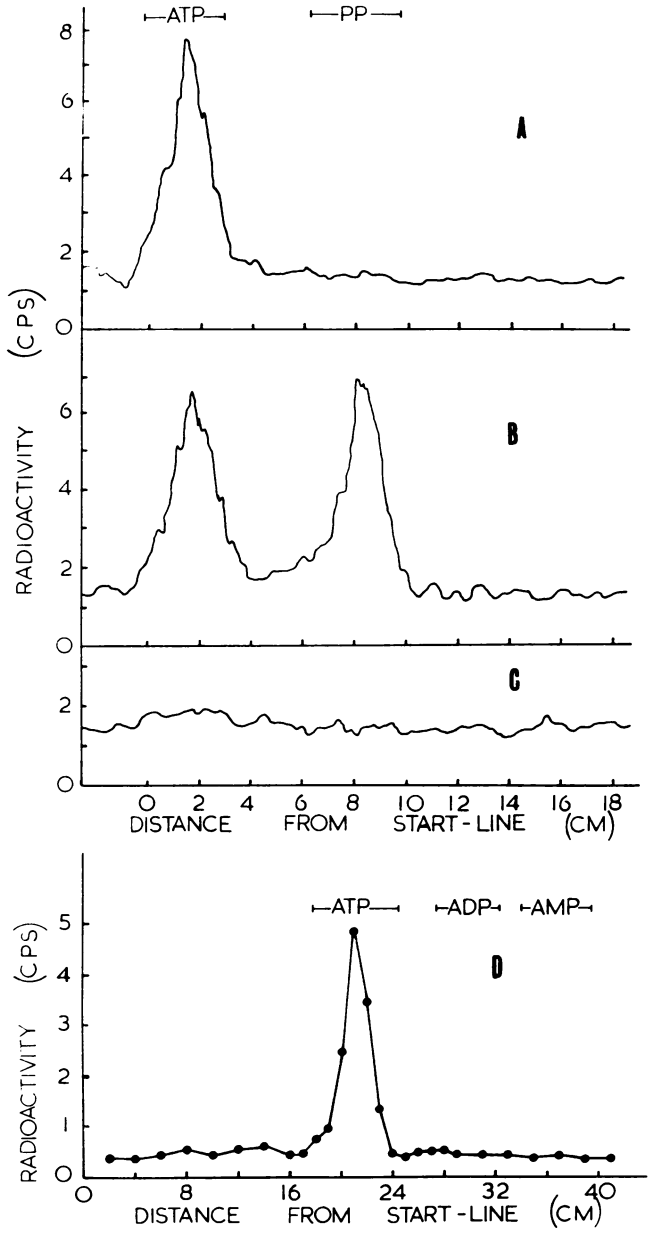


FIG. 7. Radiochromatogram traces of material eluted from charcoal with 0.1 M NH_3 in 50% (v/v) ethanol following standard assays. A: With sulfate using solvent I; C: without sulfate using solvent I. B: Chromatography of material eluted from charcoal following a standard assay containing sulfate together with added ^{32}P-pyrophosphate using solvent I. D: Chromatography of material eluted from charcoal following a standard assay containing sulfate using solvent III. The same dialyzed extract was used throughout.

fluoride was sufficient to account for the lower sulfate-dependent pyrophosphate exchange observed when fluoride was omitted from assays of ATP sulfurylase (Fig. 6). Sulfate-dependent pyrophosphate exchange in the presence of fluoride was approximately maximal at 2 to 4 mM pyrophosphate and decreased at higher concentrations. In the absence of fluoride, however, sulfate-dependent pyrophosphate exchange at low concentrations of pyrophosphate was much less than the exchange observed with fluoride. Sulfate-dependent pyrophosphate exchange in the absence of fluoride, relative to the exchange with fluoride, gradually increased as the concentration of pyrophosphate was increased until at 4 mM the two exchange rates were approximately equal

Table I. *Effect of Sodium Fluoride upon the Activities of ATPase and Pyrophosphatase of Spinach Leaf Tissue under Conditions Analogous to the Standard Assay of ATP Sulfurylase*

The amount of ATP and pyrophosphate used as substrate for the respective enzymes was 2 μmoles. The enzyme extract used in this experiment was the same as that used to investigate the effect of pyrophosphate concentration upon ATP sulfurylase (Fig. 6).

	ATPase	Pyrophosphatase
	μmoles P_i produced/assay/15 min	
Without fluoride	0.04	3.75
With 10 mM fluoride	0.01	0.00

(Fig. 6). This observation confirms that the main effect of fluoride was to inhibit pyrophosphatase activity. The optimal concentration of fluoride required for maximal sulfate-dependent pyrophosphate exchange in the standard assay was 5 to 20 mM. Considerable variation in the fluoride stimulation of sulfate-dependent pyrophosphate exchange with the standard assay was observed ranging from 1.2- to 3-fold; this variation was presumably caused by variation in pyrophosphatase activity between different batches of tissue.

The method described in this paper provides a simple, rapid, and sensitive assay for measuring ATP sulfurylase. The principle of the method is analogous to the pyrophosphate exchange reaction used in the study of aminoacyl-tRNA synthetases; the reaction is studied by measuring the sulfate-dependent incorporation of pyrophosphate into ATP by the back reaction while the system comes to equilibrium:

$$ATP + SO_4^{2-} \xrightleftharpoons{Mg^{2+}} APS + PP_i$$

The very nature of this reaction and the high activity of ATP sulfurylase in spinach leaf tissue emphasizes the warning made by Marcus (11) on the danger of using magnesium sulfate as a source of Mg^{2+} in other pyrophosphate exchange reactions (thiokinases, aminoacyl-tRNA synthetases etc.) employing the ^{32}P-pyrophosphate exchange technique. The method described in this paper for the assay of ATP sulfurylase can be made as sensitive as the operator desires by adjusting the specific radioactivity of the ^{32}P-pyrophosphate. This contrasts sharply with the molybdate method in which the sensitivity is governed by the sensitivity of the estimation of phosphate. The method described in this paper for instance, is approximately 500 times more sensitive than the molybdate method. The pyrophosphate exchange assay should prove a very useful technique for studies on the purification, substrate specificity, and detailed kinetics of this important enzyme.

Acknowledgment—W. H. Shaw was the holder of a La Trobe University Research Scholarship. We are indebted to the Botany Department, University of Melbourne, for providing some of the facilities for the radiochromatography.

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