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MULTIPLE FORMS OF α-GLUCAN PHOSPHORYLASE IN BANANA FRUITS: PROPERTIES AND KINETICS

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Abstract—Multiple forms of α-glucan phosphorylase isolated from mature banana fruit pulp differ from each other in several respects—pH optimum, temperature optimum, energy of activation, primer specificity, kinetics, and sensitivity towards metal ions, nucleotides, sugar mucleotides, amino acids, glycolytic intermediates, sulfhydryl binding agents, sulfhydryl reagents and phenolics.

INTRODUCTION

The occurrence of multiple forms of phosphorylase has been demonstrated in several animal [1–4] and plant tissues [5–12]. In animal tissue they exist in two forms, one an ATP independent "active form" and the other an AMP dependent "inactive form" [2–4]. The interconversion of these forms regulates the degradation of glycogen [2–4]. In plants such a mechanism has not been reported. Instead the regulation of starch metabolism could occur by multiplicity of α-glucan phosphorylase. In a previous paper [12] characterization and separation of multiple forms of α-glucan phosphorylase from mature and immature banana fruit pulp were described. One of the forms was allosteric in nature and thought to regulate starch metabolism [13]. The present communication presents studies on the three forms of the enzyme from mature banana fruit pulp.

RESULTS

The following studies were carried out on three multiple forms of phosphorylase (A, B and C), isolated from mature banana fruit pulp [12], under conditions of linearity with respect to enzyme concentration and time.

Freedom from contaminating enzymes

All three enzyme fractions were free from phosphoglucomutase, glucosephosphate isomerase, unspecific phosphatase, ATPase, branching enzyme, amylase, all tested at the optimum pH of phosphorylase. No activity for Q-enzyme could be detected at pH 6, under the conditions of phosphorylase assay or at pH 8 in fractions containing phosphorylase A or phosphorylase B activity. The Q-enzyme activity was detected in the fraction containing phosphorylase C activity, tested at pH 5.5 with amylose as a substrate. The enzyme activity, however, could not be detected with amylopectin or starch as a substrate.

Enzyme stability

All three fractions were stable for at least one month when stored at 0–4°. Phosphorylase A and phosphorylase C were also stable at −18°. Freezing of phosphorylase B for 24 hr at −20° and thawing once led to 34% loss in its activity. Dialysis of phosphorylase A and phosphorylase C for 24 hr against water did not alter the activity. However, phosphorylase B activity was completely lost on dialysis against water for 12–16 hr at 0–4°. The activity was not restored when the enzyme preparation was preincubated with 5 mM pyridoxal phosphate, AMP or ATP or 10 mM cysteine. Addition of 0.3 M $(NH_4)_2SO_4$, Na_2SO_4, $MgSO_4$ and NaCl also did not restore the enzyme activity.

pH–activity relationship

The enzyme activity was determined using 0.5 M citrate buffer in the pH range 4–6.2 and 0.5 M Tris–HCl buffer in the range 7.2–9.5. The optimum activity of phosphorylase A and phosphorylase C was found at pH 5 and 5.5 respectively. Phosphorylase B, on the other hand, showed broad pH optimum in the range 5.5–6.2. Lee [14] reported a sharp pH optimum at 6.5 for purified potato phosphorylase. The following experiments with phosphorylase A, B and C were carried out at pH 5, 6 and 5.5 respectively.

Primer specificity

In addition to soluble starch, the three fractions could utilize dextrin. As compared to soluble starch, dextrin though equally active for phosphorylase B on an equal weight basis was 12 and 50% less active for phosphorylase A and phosphorylase C resp. (Table 1). Phosphorylase A and phosphorylase C could utilize glycogen as a primer though less efficiently than starch but phosphorylase B was completely inactive with glycogen. In contrast to phosphorylase B, phosphorylase A and phosphorylase C could utilize achrodextrin and maltose as primers. None of the three forms exhibited activity in the absence of primer.

Identification of product

When maltose or achrodextrin was used as a primer, the product of phosphorylase A reaction gave a blue coloration with iodine (maximum at *ca* 650 nm). The product thus appears to be α-1,4-glucan. However, the

Table 1. Primer specificity

Primer	Phosphorylase A	Phosphorylase B	Phosphorylase C
	Activity, units/ml		
Nil	Nil	Nil	Nil
Starch	23.0	3.7	11.0
Glycogen (Oyster)	6.4	Nil	2.3
Dextrin	20.2	3.7	5.5
Achrodextrin	12.9	Nil	8.3
Maltose	10.6	Nil	7.8

One mg of polysaccharide or maltose was used in the assay system. Achrodextrin was prepared from soluble starch as described by Porter [37].

product of phosphorylase C reaction with maltose or achrodextrin as a primer, gave a purple coloration with a broad peak in the range 530–560 nm. The product appears to be amylopectin. This is understandable since phosphorylase C fraction also contains Q-enzyme.

Test for phosphorylase in the direction of starch degradation

To test the reaction in the direction of starch degradation, the enzyme activity was followed by the disappearance of the starch–iodine complex. The results indicate that the reaction was linear for at least 30 min with the three forms of phosphorylase.

Influence of temperature

The reaction was initiated by the addition of enzyme and not the substrate. The reaction velocity increased with rising temperature and optimum activity of phosphorylase forms A, B and C were obtained at 45°, 35° and 50° resp. (Fig. 1).

For calculating the energy of activation the values for logarithm of reaction velocity against reciprocal of absolute temperature was plotted. Phosphorylase A and phosphorylase C showed a sharp discontinuity at 35° and thus gave two distinct lines for each forms of the enzyme. Both of these forms were stable when stored at 45° for 30 min. For phosphorylase A, the E values were 20240 cal/mol between 15 and 35° and 6440 cal/mol between 35 and 45°. The E values for phosphorylase C

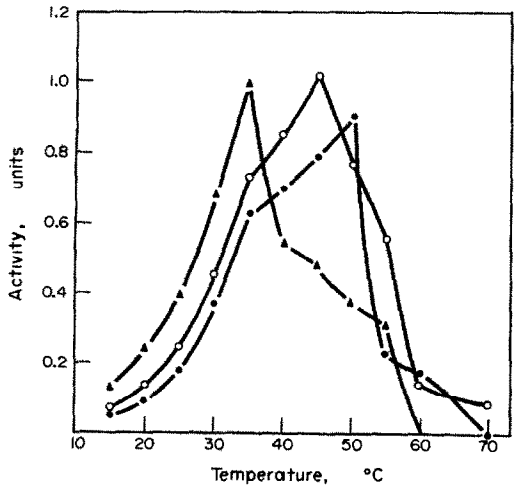

Fig. 1.

were 22080 cal/mol between 15 and 35° and 4600 cal/mol between 35 and 50°. Phosphorylase B, however, did not show a break in the plot and gave an E value of 16600 cal/mol between 15 and 35°. At temperatures above 35°, there was inactivation of the enzyme.

Effect of metal ions and of chelating agents

The effect of $MgCl_2$, $CaCl_2$ and $CuSO_4$ and of metal chelating agent, EDTA, was tested at 1 and 5 mM concn. Mg^{2+}, Ca^{2+} and Cu^{2+} did not produce any significant inhibition or activation. EDTA at 5 mM concn produced 46% activation of phosphorylase C, but activities of phosphorylase A and phosphorylase B were not significantly activated. The activity of phosphorylase C was not influenced by Hg^{2+} but the activity of phosphorylase A was inhibited 21% at 0.05 mM concn. Phosphorylase B was most sensitive and 73% inhibition occurred at 0.05 mM $HgCl_2$.

Effect of glycolytic intermediates and of D-glucose

The effect of glycolytic intermediates, fructose 1,6-P_2, glucose-6-P and 3-phosphoglycerate and of D-glucose was studied at 1 and 5 mM concn. None of these compounds inhibited or activated any form.

Effect of AMP, ATP and of pyridoxal phosphate

AMP and ATP did not show significant effect on phosphorylase A and phosphorylase C activities when tested in 1 and 5 mM concn. Phosphorylase B was slightly inhibited by AMP; at 5 mM AMP the inhibition was 18%. ATP proved to be a potent inhibitor of phosphorylase B and inhibited 20 and 90% at 1 and 5 mM resp. Pyridoxal phosphate did not significantly alter the activity of any of the three forms of phosphorylase.

Effect of amino acids

DL-Serine, DL-leucine, DL-isoleucine, DL-methionine, DL-ornithine and L-arginine did not activate or inhibit the rate of reaction of any of the three forms of phosphorylase when present in assay system at a final concn of 1 and 5 mM. In contrast to phosphorylase A and phosphorylase C, which were unaffected also by aromatic amino acids, phosphorylase B showed 48, 33 and 41% inhibition in the presence of L-tyrosine, DL-phenylalanine and DL-tryptophan respectively, when tested at 1 mM concn; increasing the concn of amino acids to 5 mM resulted in 90–97% inhibition.

Effect of sulfhydryl binding agents and of sulfhydryl reagents

None of the three forms of phosphorylase was inhibited by 1 mM *p*-chloromercuribenzoate (*p*CMB) in the assay system. Preincubation of the enzyme with 1 mM *p*CMB for 90 min at 30° did not significantly affect phosphorylase A and phosphorylase C activities but the activity of phosphorylase B was inhibited by 34%. Iodoacetate in 1 or 5 concn and *N*-ethylamaleimide in 0.5 and 1 mM concn did not inhibit the activity of any of the three enzymic forms.

Sulfhydryl reagents, such as L-cysteine, β-mercaptoethanol or reduced glutathione did not influence the enzyme activity when tested in 1 and 10 mM concn.

Effect of sugar nucleotides

UDP-Glucose inhibited the activities of phosphorylase A, phosphorylase B and phosphorylase C by 43, 27 and

Table 2. Effect of sugar nucleotides

Sugar nucleotide	Concentration (mM)	Phosphorylase A Activity, units/ml	B	C
Nil		20.2	3.9	11.0
UDP–Glucose	1	11.4	2.8	9.1
	10	6.0	0.9	8.0
ADP–Glucose	1	17.0	3.5	9.1
	5	11.0	3.0	5.2

18% respectively at 1 mM concn; increasing the concn to 10 mM resulted in 70–74% inhibition of phosphorylase A and phosphorylase B, but phosphorylase C was inhibited only 27% (Table 2). ADP-Glucose showed slight inhibition (10–17%) at 1 mM concn, but at 5 mM concn the sugar nucleotide inhibited phosphorylase A, phosphorylase B and phosphorylase C by 45, 21 and 52% respectively.

Effect of phenolic compounds

These studies were carried out by keeping the protein concn of the assay system equal, either by taking enzyme containing the same amount of protein in the assay system or by externally adding bovine serum albumin. Simple monohydroxyphenols in 10 mM concn inhibited phosphorylase A, phosphorylase B and phosphorylase C by 50, 40 and 65% respecitvely. Resorcinol, a dihydroxyphenol, when used in 1 mM concn inhibited phosphorylase A and phosphorylase B by 50% but phosphorylase C was inhibited by only 23%. Another dihydroxyphenol, pyrocatechol, in 10 mM concn inhibited phosphorylase A and phosphorylase C by 50 and 45% respectively, but the activity of phosphorylase B was inhibited 73%. The trihydroxyphenol, phloroglucinol, in 1 mM concn produced 13 and and 20% inhibition of phosphorylase A and phosphorylase C respectively, but phosphorylase B was inhibited 40%.

Tannic acid (5 μg) completely inhibited phosphorylase B but phosphorylase A and phosphorylase C were inhibited only 63 and 50% respectively. No significant inhibition or activation of phosphorylase A and phosphorylase B was observed in the presence of *p*-coumaric acid, caffeic acid and cinnamic acid tested in 1 and 10 mM concn. The activity of phosphorylase C was not markedly altered in the presence of caffeic acid and *p*-coumaric acid, but cinnamic acid produced 25% inhibition in 1 mM concn. The glycoside, phloridzin, in 10 mM concn inhibited phosphorylase A, phosphorylase B and phosphorylase C by 86, 69 and 76% respectively.

The effect of primer and substrate concentration

The substrate saturation curves for glucose-1-phosphate with different primers as well as the primer saturation curves at a fixed concn of glucose-1-phosphate were hyperbolic in nature for all the three forms of phosphorylase. The kinetic parameters are summarized in Table 3.

The data indicate highest affinity of phosphorylase A for glucose-1-phosphate when starch was the primer, followed by maltose and glycogen. The Pi liberation as well as the formation of amylose and the low K_m value for maltose compared to glycogen indicate that maltose acts as a good primer for phosphorylase A. At a fixed concn of glucose-1-phosphate, the data indicates the lowest K_m value for starch followed by maltose and glycogen. Maltose also acts as a good primer for phosphorylase C, although the affinity for glucose-1-phosphate was higher in the presence of starch compared to maltose.

The K_m value for glucose-1-phosphate with starch as a primer was lowest for phosphorylase A, followed by phosphorylase C and phosphorylase B.

DISCUSSION

Mature banana fruit pulp contains three forms of starch phosphorylase, which are separable by ammonium sulfate fractionation and DEAE-cellulose chromatography [12]. These forms can be distinguished on the basis of some of the properties. Differences were observed in optimum pH, optimum temperature, stability at different temperature, primer specificity, response towards EDTA and Hg^{2+}, and inhibition by nucleotides, aromatic amino acids and phenolics.

The energy of activation of glycogen phosphorylase from rabbit muscle is 21 200 cal/mol [15]. In contrast phosphorylase A and phosphorylase C from banana fruit gave two values for energy of activation. The two energy

Table 3. Kinetic parameters of multiple forms of α-glucan phosphorylase

Phosphorylase	Substrate or primer	K_m	V_{max}, μmol Pi formed/ 30 min/mg protein
A	Glucose-1-phosphate (starch)	1.4 mM	2081
	Glucose-1-phosphate (glycogen)	5.0 mM	831
	Glucose-1-phosphate (maltose)*	2.2 mM	1130
	Glucose-1-phosphate (maltose)†	4.2 mM	
	Starch	0.24 g/l	1560
	Glycogen	1.1 g/l	781
	Maltose	0.66 g/l	1250
B	Glucose-1-phosphate (starch)	3.3 mM	775
	Starch	0.95 g/l	775
C	Glucose-1-phosphate (starch)	2.7 mM	500
	Glucose-1-phosphate (maltose)	4.0 mM	415
	Starch	1.21 g/l	625
	Maltose	1.33 g/l	415

* Enzyme assayed by Pi liberation. †Enzyme assayed by amylose formation. Fixed concentration of glucose-1-phosphate was 3.3 mM and primer was 1 mg/assay system.

of activation with transition occurring at 35° may not be due to the decreased stability of the enzyme, since both the preparation were stable when tested at 45° for 30 min. These forms of the enzyme may have two active conformational states with different activation energy and the discontinuity in the Arrhenius plot may result from a reversible transition from one state to another. Amylases, mitochondrial and some other enzymes show this phenomenon of two activation energies [16–19].

Phosphorylase A and phosphorylase C utilized glycogen, achrodextrin or maltose as a primer, in addition to starch and dextrin. The K_m value of phosphorylase A for glycogen (1.1 g/l.) was more than that for starch (0.24 g/l.) and for maltose (0.66 g/l.), indicating lower affinity for glycogen. Phosphorylase B was inactive with glycogen, achrodextrin or maltose as a primer. Glycogen serves as a primer for some plant phosphorylases [20, 21] but its utilization by potato enzyme has been questioned by Fischer and Helpert [22], who observed glycogen as a primer only in the presence of trace contamination of starch type polysaccharide. Gerbrandy and Doorgeest [8] reported two isoenzymes of phosphorylase from potato tubers which were active with glycogen. While saccharides of higher degree of polymerization are utilized by phosphorylase B, phosphorylase A and phosphorylase C could also use malto oligosaccharides of low degree of polymerization. The complete absence of catalytic activity of the three forms of the enzyme without primer indicates the absence of primer-less form of phosphorylase in banana.

A striking difference between three forms of the enzyme was the sensitivity towards aromatic amino acids. Whereas, phosphorylase B activity was powerfully inhibited by phenylalanine, tyrosine and tryptophan, phosphorylase A and phosphorylase C activities remained unaltered in the presence of amino acids. Blank and Sondheimer [23] found the activity of potato phosphorylase unaltered or slightly elevated in the presence of 1 mM tyrosine.

There were also similarities amongst the three forms of starch phosphorylase. All the forms exhibited activity with starch or dextrin as a primer. The substrate saturation curves for glucose-1-P in the presence of starch or other primers were hyperbolic in shape indicating classical kinetic. None of the glycolytic intermediates tested inhibited or activated any of the forms of phosphorylase. D-Glucose is known to inhibit potato phosphorylase [22]. In contrast, none of the forms of banana phosphorylase was inhibited by D-glucose. All the three forms of banana phosphorylase were inhibited by UDP-glucose and ADP-glucose. The sulfhydryl finding agents or the sulfhydryl reagents did not show any significant effect when added in the assay system of any of the three forms of phosphorylase. It appears that sulfhydryl groups are not involved in enzyme catalysis. It is pertinent to note that potato phosphorylase is inactivated by *p*CMB and contains six moles of -SH groups per mole of protein [14]. All the three forms were sensitive to phloridzin and therefore, resemble muscle phosphorylase [24] and potato phosphorylase [25]. In this respect Fischer and Hilpert [22] reported that potato phosphorylase in its pure form was not inhibited by phloridzin.

The fact that phosphorylase A is absent in immature fruit and appeared in mature fruit [12], that phosphorylase B has allosteric properties [13] and that differences existed in other properties of the three forms of the enzyme suggested different physiological roles for the multiple forms of phosphorylase in banana fruit.

EXPERIMENTAL

Plant. Banana fingers (*Musa paradisiaca*, cooking variety) were collected immediately before use from plants grown in the departmental garden. Banana fingers, 60–65 days old, having a pulp to peel ration of 1.3–1.5 were used.

Enzyme assay. α-Glucan phosphorylase. Enzyme activity was assayed as described earlier [12]. NaF (20 mM) was added to inhibit phosphatase activity. One unit of the enzyme was equivalent to the liberation of 1 μmol of Pi in 30 min at 30° under the experimental conditions. In some experiments the enzyme was assayed by determining the polysaccharide formed, using maltose as a primer. The enzyme assay was carried out as above, but the reaction was terminated on heating at 100° for 30 sec and amylose formed was determined according to ref. (26), using the I_2 reagent. The unit of phosphorylase was expressed as 1 μg equiv of soluble starch under the experimental conditions. Sp. act. of the enzyme was expressed as units per mg protein.

The phosphorylase in the direction of starch degradation was followed by the disappearance of the starch–I_2 colour complex. The incubation mixture contained 0.5 ml of 1% soluble starch, 2 ml of 0.2 M Pi buffer, pH 6.2, 0.6 ml of 0.2 M NaF, enzyme and H_2O to 6 ml. While the reaction mixtures were incubated at 30°, 1 ml aliquots were removed at the time indicated and mixed with 0.1 ml KI/I_2 reagent. The resulting soln was diluted to 10 ml with H_2O and read against a reagent blank (660 nm).

Other enzymes. The following enzymes were assayed under optimum conditions and also under the assay conditions of α-glucan phosphorylase. Phosphoglucomutase and phosphohexoisomerase were assayed according to ref [27]; unspecific phosphatase according to ref [28] using glucose-1-phosphate or β-glycerophosphate, as the substrate; ATPase according to ref [29]; Q-enzyme according to ref [30]; and amylases according to ref [31] based on reducing sugar estimation and also according to ref [32] by measuring the disappearance of the blue colour complex using amylose, amylopectin or starch as a substrate. The assay of branching enzyme was carried out according to ref [33], but at pH 6 and using amylose or amylopectin as a substrate. The assay was also carried out according to ref [34] using 80 mM glucose-1-P adjusted to pH 6.

Protein determination. The TCA protein ppts were washed according to ref [35] and protein estimated according to ref [36]. Bovine serum albumin was used as a standard.

Purification of α-glucan phosphorylase. Three forms of α-glucan phosphorylase were isolated from mature banana pulp as described in ref [12].

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REFERENCES

1. Brown, D. H. and Cori, C. F. (1961) Animal and plant polysaccharide phosphorylases. In *The Enzymes* (Boyer, P. D., Lardy, H. and Myrback, K. eds.) Vol. 5, pp. 207–28. Academic Press, New York.
2. Krebs, E. G. and Fischer, E. H. (1962) in *Advances in Enzymology* (Nord, F. F. ed.) Vol. 24, pp. 263–87. Interscience New York.
3. Krebs, E. G., Delange, R. J., Kemp, R. G. and Riley, W. D. (1966) *Pharmacol. Rev.* **18**, 163.
4. Fischer, E. H. and Krebs, E. G. (1966) *Fed. Proc. Fed. Am. Socs. Exp. Biol.* **25**, 1511.
5. DeFekete, M. A. R. (1968) *Planta* **79**, 208.

6. Slabnik, E. and Frydman, R. B. (1970) *Biochem. Biophys. Res. Commun.* **38,** 709.
7. Gerbrandy, Sj. J. and Verleur, J. D. (1971) *Phytochemistry* **10,** 261.
8. Gerbrandy, Sj. J. and Doorgeest, A. (1972) *Phytochemistry* **11,** 2403.
9. Frederick, J. F. (1962) *Phytochemistry* **1,** 153.
10. Frederick, J. F. (1967) *Phytochemistry* **6,** 1041.
11. Tsai, C. Y. and Nelson, O. E. (1968) *Plant. Physiol.* **43,** 103.
12. Singh, S. and Sanwal, G. G. (1975) *Phytochemistry* **14,** 113.
13. Singh, S. and Sanwal, G. G. (1973) *Biochim. Biophys. Acta* **309,** 280.
14. Lee, Y. P. (1960) *Biochim. Biophys. Acta* **43,** 25.
15. Madsen, N. B. and Cori, C. F. (1954) *Biochim. Biophys. Acta* **15,** 516.
16. Massey, V., Curti, B. and Gunther, H. (1966) *J. Biol. Chem.* **241,** 2347.
17. Robyt, J. F. and Ackerman, R. J. (1971) *Arch. Biochem. Biophys.* **145,** 105.
18. Lenaz, G., Sechi, A. N., Parenti-Castelli, G., Landi, L. and Bertoli, E. (1972) *Biochem. Biophys. Res. Commun.* **49,** 536.
19. Orengo, A. and Saunders, G. F. (1972) *Biochemistry* **10,** 1761.
20. Green, D. E. and Stumpf, P. K. (1942). *J. Biol. Chem.* **142,** 355.
21. Sumner, J. B., Somers, G. F. and Sisler, E. (1944) *J. Biol. Chem.* **152,** 479.
22. Fischer, H. and Hilpert, H. M. (1953) *Experimentia* **9,** 176.
23. Blank, G. E. and Sondheimer, E. (1969) *Phytochemistry* **8,** 823.
24. Cori, C. F., Cori, G. T. and Green, A. A. (1943) *J. Biol. Chem.* **151,** 39.
25. Nakamura, M. (1952) *J. Agric. Chem. Soc. (Japan)* **26,** 267.
26. McCready, R. M. and Hassid, W. Z. (1943) *J. Am. Chem. Soc.* **65,** 1154.
27. Gibbs, M. and Turner, J. F. (1964) in *Modern Methods of Plant Analysis* (Linsken, H. F., Sanwal, B. D. and Tracey, M. Y. eds.) Vol. 7, pp. 520–45. Springer-Verlag, Berlin.
28. Heppel, L. A. (1955) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O. eds.) Vol. 2, pp. 530–33. Academic Press, New York.
29. Kielley, W. W. (1955) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O. eds.) Vol. 2, pp. 593–95. Academic Press, New York.
30. Feingold, D. S., Neufeld, E. F. and Hassid, W. Z. (1964) in *Modern Methods of Plant Analysis* (Linskens, H. F., Sanwal, B. D. and Tracey, M. V. eds.) Vol. 7, 474–519.
31. Bernfield, P. (1955) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O. eds.) Vol. 1, pp. 149–58. Academic Press, New York.
32. Swain, R. R. and Dekkar, E. E. (1966) *Biochim. Biophys. Acta* **122,** 75.
33. Larner, J. (1955) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O. eds.) Vol. 1, pp. 222–25. Academic Press, New York.
34. Gibson, W. B., Brown, B. I. and Brown, D. H. (1971) *Biochemistry* **10,** 4253.
35. Khanna, S. K., Mattoo, R. L., Viswanathan, P. N. Tewari, C. P. and Sanwal, G. G. (1969). *Indian J. Biochem.* **6,** 21.
36. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.
37. Porter, H. K. (1950) *Biochem. J.* **47,** 476.