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CHANGES IN 5'-NUCLEOTIDASE AND GLUCOSE-6-PHOSPHATASE OF *PHASEOLUS VULGARIS* COTYLEDON TISSUE DURING GERMINATION

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Abstract—Experiments with cotyledon tissue from *Phaseolus vulgaris* have shown that 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, E.C. 3.1.3.5) and glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, E.C. 3.1.3.9) activities increase during germination to reach a peak at 5 days of age and thereafter decline as the cotyledons become senescent. Cell fractionation studies of 4-day-old tissue showed that the enzymes are distributed in parallel among sedimentable subcellular fractions, 50–60% of the particulate activities being present in the microsomal fraction. This has been interpreted as indicating that both enzymes are associated with endoplasmic reticulum in the intact cell and thus the increased activities of the enzymes in the early stages of germination may well reflect in part a proliferation of endoplasmic reticulum at this time. The much reduced activities in senescent tissue are consistent with the digestion of cell cytoplasm that occurs in the late stages of germination. Both enzymes are also present in isolated soluble fractions in proportions which increase with advancing senescence. In 7- and 9-day-old tissue about 90% of the activity of each enzyme is soluble. This observation is discussed in terms of a possible inactivation or solubilization of the bound forms of the enzymes during the late stages of germination.

INTRODUCTION

A NUMBER of well defined alterations in cellular metabolism take place in cotyledon tissue during germination. Mitochondrial respiratory activity markedly increases during the initial stages, but after attaining a peak, declines.[1–3] In cotyledons of *Phaseolus vulgaris* this decrease is coincident with changes in mitochondrial morphology such as swollen intercrystal spaces and a darkened matrix, conditions which may indicate an initiated breakdown of mitochondrial structure.[3] Isocitrate lyase (isocitrate glyoxylate-lyase, E.C. 4.1.3.1) and malate synthetase (L-malate glyoxylate-lyase, E.C. 4.1.3.2), key enzymes of the glyoxylate cycle, also show increased activity in castor bean seeds during germination. Peak levels coincide with a maximal conversion of fat to carbohydrate in the endosperm and thereafter the activities decrease.[4–5] These two enzymes together with those for β-oxidation are sequestered within glyoxysomes and recent evidence has attributed the rise and fall in their activities to successive synthesis and destruction of this organelle during germination.[6] In pea cotyledons enzymes of carbohydrate metabolism such as hexokinase

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(ATP: D-hexose-6-phosphotransferase, E.C. 2.7.1.1), glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, E.C. 1.1.1.49), 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate: NADP oxidoreductase, E.C. 1.1.1.43), and phosphoglucose isomerase (D-glucose-6-phosphate ketol-isomerase, E.C. 5.3.1.9) also exhibit the rise and decline in activity characteristic of cotyledon tissue during germination.[7] Although increased enzyme activities during this period have in some instances been shown to reflect *de novo* protein synthesis,[7,8] in this case[7] a correlation between *in vivo* substrate and cofactor levels and alterations in enzyme activity was demonstrated. This correlation has been interpreted as indicating that substrate and cofactor concentrations may influence subsequent changes in enzyme activity.[7]

It is well documented that hydrolytic activity increases in cotyledon tissue during germination. Decreased levels of RNA during germination in cotyledons of *Pisum arvense* and *Arachis hypogaea* have been attributed at least in part to corresponding increases in ribonuclease activity.[9,10] Polysaccharide hydrolysis during germination is implied in the observation that for cotyledons of *Phaseolus vulgaris* the specific activities of various glycosidases increase and subsequently decline.[11] The disappearance of storage bodies from the cells of cotyledons, in particular protein bodies, has been observed as a prominent feature of germination[12,13] and this also implies hydrolytic activity. For *Phaseolus vulgaris*, it is clear that the ultimate effect of the increased hydrolytic activity accompanying germination is total cytoplasmic degradation by the late stages of germination.[13]

In this study the effects of germination on glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, E.C. 3.1.3.9) and 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, E.C. 3.1.3.5) in cotyledon tissue of *Phaseolus vulgaris* were investigated. It seems quite clear for several mammalian tissues that 5′-nucleotidase is localized on the plasma membrane[14–16] and glucose-6-phosphatase on endoplasmic reticulum.[17] For plant tissue their subcellular distributions are much less clearly resolved. By enzyme histochemistry at the light microscope level, it has been shown that both glucose-6-phosphatase and 5′-nucleotidase are present in the cotyledons of *Pisum arvense* but low resolution precluded an assessment of their structural localizations within the cell.[18] Glucose-6-phosphatase has been detected in microsomal fractions from *Vigna sinensis*[19] and from cotyledon tissue of *Phaseolus vulgaris*.[20] However, this enzyme is also present as a soluble activity in *Phaseolus vulgaris* cotyledons in proportions which increase with advancing senescence of the tissue.[20] Thus, in addition to determining the effects of germination on the total levels and specific activities of glucose-6-phosphatase and 5′-nucleotidase, changes in the subcellular distribution of the two enzymes with advancing senescence were also compared.

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RESULTS

Changes in total activity and in specific activity of glucose-6-phosphatase and 5′-nucleotidase during germination are illustrated in Figs. 1 and 2 respectively. The total activities were relatively low in 1-day-old tissue but subsequently increased to reach a peak at 5 days of age. By this stage the cotyledons were beginning to show visible signs of senescence and thereafter, the enzyme activities progressively decreased such that levels in 10-day-old tissues were comparable in magnitude to those of 1-day-old tissue (Fig. 1). There was a progressive rise in specific activities of the enzymes during development, although in each case, a peak was manifest in 8-day-old tissue (Fig. 2). It is to be noted that in spite of these reasonably large scale fluctuations in activity, the patterns of change for glucose-6-phosphatase and 5′-nucleotidase remain quite parallel throughout the entire life span of the cotyledon.

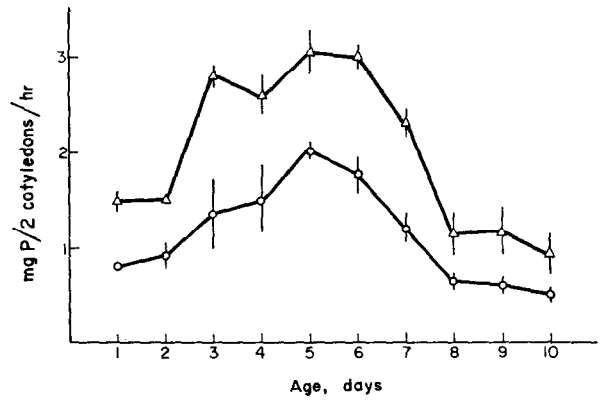

FIG. 1. CHANGES IN TOTAL 5′-NUCLEOTIDASE AND GLUCOSE-6-PHOSPHATASE ACTIVITIES IN *Phaseolus vulgaris* COTYLEDONS DURING GERMINATION.
(○) 5′-Nucleotidase; (△) glucose-6-phosphatase. Standard errors of the means are indicated by vertical lines; $n = 3$ or 4.

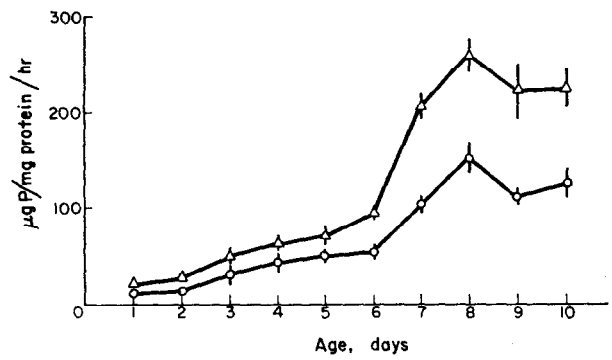

FIG. 2. CHANGES IN SPECIFIC ACTIVITIES OF 5′-NUCLEOTIDASE AND GLUCOSE-6-PHOSPHATASE IN *Phaseolus vulgaris* COTYLEDONS DURING GERMINATION.
(○) 5′-Nucleotidase; (△) glucose-6-phosphatase. Standard errors of the means are indicated by vertical lines; $n = 3$ or 4.

The distributions of glucose-6-phosphatase and 5′-nucleotidase among subcellular particulate fractions isolated from a homogenate of 4-day-old cotyledon tissue also proved to be parallel; levels of the enzyme activities were closely similar in each of the cytoplasmic fractions, particularly within any one experiment (Table 1). Recoveries ranged from 94–108% for glucose-6-phosphatase and 65–110% for 5′-nucleotidase (Table 1). For both enzymes the greatest proportion (50–60%) of the particulate activity sedimented with the microsomal fraction (Table 1).

TABLE 1. LEVELS OF GLUCOSE-6-PHOSPHATASE AND 5′-NUCLEOTIDASE IN SUBCELLULAR FRACTIONS FROM 4-day-old COTYLEDON TISSUE OF *Phaseolus vulgaris*

	A		B		C	
	G-6-Pase	5′-Nuc-ase	G-6-Pase	5′-Nuc-ase	G-6-Pase	5′-Nuc-ase
Nuclear	12·5	10·0	16·6	11·3	16·0	14·3
Mitochondrial	3·7	5·0	6·5	5·0	6·3	6·6
Microsomal	17·5	24·5	22·5	24·1	28·2	28·3
Recovery (%)	94·0	83·0	108·0	110·0	94·0	65·0

Activities in the fractions are expressed as a percentage of recovered activity. G-6-Pase = glucose-6-phosphatase. 5′-Nuc-ase = 5′-nucleotidase. A, B and C refer to three separate experiments.

It has been previously reported for this tissue that glucose-6-phosphatase is soluble in proportions which increase with advancing senescence[20] and in this study 5′-nucleotidase was also found to be present in isolated soluble fractions. Furthermore, changes in percentage solubility for the two enzymes during germination have proven to be similar (Table 2). In 2-day-old tissue approximately 70% of the total activity was soluble for each enzyme, but by 4 days of age this had decreased to about 53% for glucose-6-phosphatase and 55% for 5′-nucleotidase (Table 2). Thereafter the proportions of soluble activity increased with advancing senescence such that in 7- and 9-day-old tissue about 90%

TABLE 2. LEVELS OF SOLUBLE GLUCOSE-6-PHOSPHATASE AND 5′-NUCLEOTIDASE ACTIVITIES IN COTYLEDON TISSUE OF *Phaseolus vulgaris*

		Soluble activities expressed as percentages of recovered activity	
	Expt.	Glucose-6-phosphatase	5′-Nucleotidase
2-Day-old tissue	A	74·6	69·0
	B	70·5	73·2
4-Day-old tissue	A	49·5	51·0
	B	55·5	60·0
7-Day-old tissue	A	85·9	93·5
	B	88·0	85·6
9-Day-old tissue	A	94·5	87·5
	B	92	85·7

of the total activity was soluble for both enzymes (Table 2). The cotyledons were visibly wrinkled and thus clearly senescent at this stage. This increasing proportion of soluble activity accompanying germination is further apparent from the measurements of specific activities illustrated in Table 3. For glucose-6-phosphatase and 5′-nucleotidase the specific activities in soluble fractions isolated from 7- and 9-day-old tissue were about 10 fold greater than those of the same fraction from 2-day-old tissue (Table 3). Moreover, for 2- and 4-day-old cotyledons the specific activities in the soluble fraction were roughly equal to those in a pellet containing all the particulate components of the homogenate, but were 2- to 5-fold greater than those in the pellets by days 7 and 9 (Table 3).

TABLE 3. SPECIFIC ACTIVITIES OF GLUCOSE-6-PHOSPHATASE AND 5′-NUCLEOTIDASE IN FRACTIONS FROM COTYLEDON TISSUE OF *Phaseolus vulgaris*

		Glucose-6-phosphatase		5′-Nucleotidase	
	Expt.	Pellet	Soluble fraction	Pellet	Soluble fraction
2-Day-old tissue	A	18·0	23·2	12·3	9·3
	B	16·3	19·6	10·8	12·8
4-Day-old tissue	A	55·2	75·2	8·7	8·9
	B	44·0	30·8	9·0	6·2
7-Day-old tissue	A	112·0	246·0	42·4	137·2
	B	88·0	224·0	24·0	124·8
9-Day-old tissue	A	34·2	190·0	60·0	118·2
	B	48·0	181·2	44·0	111·2

Activities are expressed as μg P/mg protein/hr. Fractions were obtained by centrifugation of homogenates at 138,000 *g* for 3 hr.

Particulate and soluble glucose-6-phosphatase activities of *Phaseolus vulgaris* cotyledons have been previously shown to have similar pH profiles[20] and in this investigation the pH profiles of microsomal and soluble 5′-nucleotidase activities were also found to be similar. They featured two optima (Fig. 3). Maximum activity occurred at pH 6·0, but there was also a small peak at pH 7·5. In a few experiments the pH 7·5 optimum was apparently masked, but in most cases it was manifest either as a small peak or as a shoulder over the range 7·0–7·5.

In order to determine whether the glucose-6-phosphatase and 5′-nucleotidase activities measurable in this tissue could be due to the action of a non-specific phosphatase, the pattern of change in total acid phosphatase activity during development was compared with those for glucose-6-phosphatase and 5′-nucleotidase. From Table 4 it is apparent that the changes for acid phosphatase are disproportionate to those for glucose-6-phosphatase and 5′-nucleotidase. Furthermore, ethylenediamine tetraacetic acid (EDTA) and potassium fluoride (KF) were routinely present in the glucose-6-phosphatase assay mixture as inhibitors of acid and alkaline phosphatases and were found to cause a 10–20% reduction in activity relative to controls in which they were not included as components of the reaction. When the same substances were included in the assay mixture for 5′-nucleotidase, no reduction in activity was apparent; in fact in some cases a slight increase was observed.

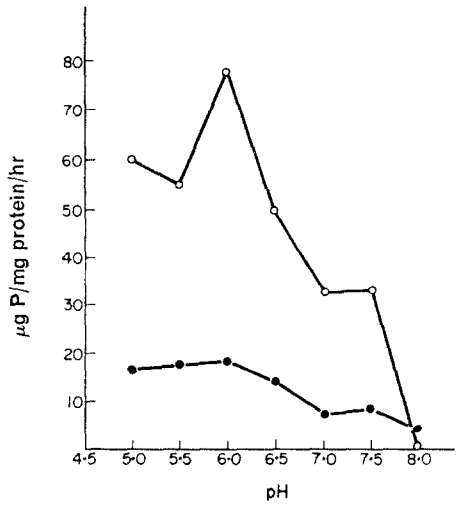

FIG. 3. pH PROFILES OF 5′-NUCLEOTIDASE FROM 4-day-old *Phaseolus vulgaris* COTYLEDONS. (○) Soluble; (●) microsomal.

TABLE 4. RELATIVE CHANGES IN ENZYME ACTIVITIES DURING DEVELOPMENT OF *Phaseolus vulgaris* COTYLEDONS

	Activity/2 cotyledons (day *n*) / Activity/2 cotyledons (day 1)		
Days after planting	G-6-Pase	5′-Nuc-ase	Acid Pase
1	1·0	1·0	1
2	1·0	1·0	2·6
3	1·9	1·7	3·8
4	1·8	1·9	4·9
5	2·1	2·5	5·3
6	2·1	2·2	4·9
7	1·5	1·5	3·1
8	0·8	0·8	1·7
9	0·8	0·8	1·0
10	0·6	0·7	0·9

n = Days 1–10 respectively. G-6-Pase = Glucose-6-phosphatase. 5′-Nuc-ase = 5′-Nucleotidase. Acid Pase = Acid Phosphatase.

DISCUSSION

It has been previously reported that in mammalian tissue glucose-6-phosphate is hydrolyzed to a small extent by acid or alkaline phosphatase.[21] This would appear to be the case in cotyledon tissue of *Phaseolus vulgaris* as well in view of the capacity of EDTA and KF, known inhibitors of acid and alkaline phosphatase in this tissue,[20] to reduce the activity of glucose-6-phosphatase by 10–20%. Fritzson[22] has reported that in rat liver tissue

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dephosphorylation of deoxyribonucleotides is accomplished primarily by non-specific acid phosphatase activity whereas ribonucleotides are acted upon largely by a specific 5′-nucleotidase. The prominent pH optimum of 6·0 that characterizes the dephosphorylation of adenosine-5′-monophosphoric acid in the cotyledon tissue suggests that the substrate can be hydrolyzed by acid phosphatase from this tissue, but there is nevertheless a smaller shoulder of activity in the pH region 7·0–7·5 that is unaffected by EDTA and KF and probably represents hydrolysis by a specific 5′-nucleotidase. The assay for 5′-nucleotidase was routinely carried out at pH 7·4. It would appear therefore that 5′-nucleotidase and glucose-6-phosphatase activities, as assayed in this investigation, are not manifestations of non-specific phosphatase activity. Indeed, alkaline phosphatase is virtually non-detectable in the tissue[20] and the totally independent fluctuations of acid phosphatase relative to glucose-6-phosphatase and 5′-nucleotidase during tissue development are not in accord with a major contribution to the hydrolysis of glucose-6-phosphate or adenosine-5′-monophosphoric acid by acid phosphatase.

The patterns of change in total activity for 5′-nucleotidase and glucose-6-phosphatase exhibit the characteristic rise and fall previously documented for mitochondrial respiration[3] and glycosidase activity[11] in bean cotyledon tissue. The rise in glucose-6-phosphatase activity during the early stages of germination implies a stimulated production of glucose, possibly from malate as formed by the glyoxylate pathway. This in turn suggests that some sugar may be translocated directly as glucose as well as in the preferred form of sucrose.[23] The increase in 5′-nucleotidase activity is consistent with the degradation of RNA in cotyledon tissue during germination, an event previously documented for *Pisum arvense*[9] and *Arachis hypogaea*.[10] Cellular autolysis, which during the earlier stages of germination involves primarily digestion of protein and starch reserves, does ultimately lead to complete degradation of the cytoplasm.[13] Thus, the dramatic fall in total levels of enzymatic activity observed in the aged tissue (Fig. 1) is not unexpected. The rising specific activities of the enzymes (Fig. 2) probably reflect progressive digestion of the protein reserves.

The subcellular distributions of glucose-6-phosphatase and 5′-nucleotidase in plant tissue contrast with those for the same enzymes in mammalian tissue. Neither glucose-6-phosphatase[24] nor 5′-nucleotidase[25] is soluble to any significant extent in rat liver tissue, and furthermore their distribution among sedimentable fractions in this tissue is distinctly non-parallel.[25] This is to be expected in view of the exclusive association of 5′nucleotidase with the plasma membrane[14–16] and of glucose-6-phosphatase with endoplasmic reticulum.[17] In bean cotyledon tissue, the parallel distribution of glucose-6-phosphatase and 5′-nucleotidase activities among sedimentable subcellular fractions suggests that the enzymes are present on the same membrane system. It seems likely that they are associated with endoplasmic reticulum since the largest proportion of the particulate activities sediments with the microsomal fraction (Table 1). Moreover it has been observed that endoplasmic reticulum is quite prominent in the cotyledon cells during the earlier stages of germination.[13] Hence the proportional rise in total levels of the enzyme activities featured in the early stages of development could very well reflect, in part, a proliferation of endoplasmic reticulum. Indeed, the proportions of soluble activity for both enzymes are lower at 4 days of age than at 2 (Table 2) implying a preferential synthesis of particulate enzymes during this period. Since non-specific phosphatases do not appear to be contributing significantly to the glucose-

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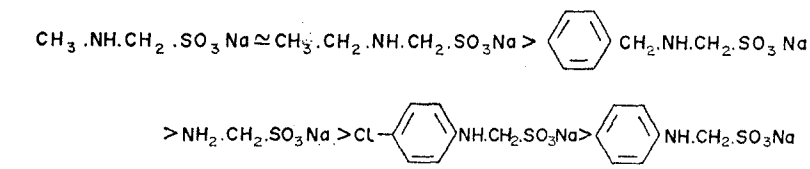
6-phosphatase and 5′-nucleotidase activities, it seems unlikely that the parallel distribution reflects the presence of a single enzyme capable of hydrolyzing both substrates.

A substantial proportion of the total activity for both enzymes is soluble by even day 2 of germination. Moreover as the cotyledons become progressively senescent these proportions increase such that in 7-day-old tissue approximately 90% of the activities are soluble. In view of the decline in total levels of both enzymes during this period, it seems likely that these increased soluble activities reflect degeneration of the particulate cytoplasm rather than *de novo* synthesis of soluble enzymes. Opik[13] has reported that the particulate elements of the cytoplasm in the storage cells of this tissue disintegrate between the fifth and eighth day of germination, an event that might be expected to cause solubilization of previously bound enzymes. A breakdown of membrane structure and an attendant solubilization of membranous enzymes is consistent with the essentially similar pH profiles of the bound and soluble forms of 5′-nucleotidase (Fig. 3) and glucose-6-phosphatase.[20] However, the increased proportions of soluble activities may also reflect partial inactivation of the bound forms of the enzymes during this period.



EXPERIMENTAL

Materials

Glucose-6-phosphate, adenosine-5′-monophosphoric acid and β-glycerophosphate were obtained from Sigma Chemical Co. All other chemicals were Fisher reagent grade.

Growth Conditions

Seeds of *Phaseolus vulgaris*, variety Kinghorn, were germinated and grown in vermiculite in the dark at 28°. The seeds were not soaked prior to planting. Cotyledons were harvested at specific ages within the period 1–10 days after planting.

Tissue Homogenization and Fractionation

Homogenates to be used for determinations of total enzyme activity per 2 cotyledons were prepared by treating the tissue with a Sorvall Omnimixer fitted with a 400 ml homogenizing cup immersed in an ice bath. For all ages of tissue 20 g of cotyledons were suspended in 60 ml of precooled 0·05 M $NaHCO_3$, pH 7·5, and homogenized with the Omnimixer at maximum speeds for 6 periods of 20 sec, each separated by 30 sec intervals for cooling. Homogenate volumes were measured after the foam caused by the treatment had settled. For 8,- 9- and 10-day-old tissues, the homogenates prepared in this manner were quite viscous and had to be diluted with an additional 30 ml of bicarbonate buffer.

Subcellular fractionations that gave rise to nuclear, mitochondrial, and microsomal fractions were performed as described previously.[20] The cotyledon tissue was homogenized in buffer consisting of 0·3 M sucrose and 0·05 M $NaHCO_3$, adjusted to pH 7·4, with a mortar and pestle. A nuclear pellet, which also contained starch and cellular debris, was obtained by centrifugation at 5000 *g* for 10 min. Mitochondrial and microsomal fractions were sedimented by centrifugation at 10,000 *g* for 15 min and 138,000 *g* for 3 hr respectively. For determinations of changes in the levels of soluble enzyme activities during development, two fractions, a pellet and a soluble fraction were obtained by direct centrifugation of homogenates at 138,000 *g* for 3 hr.

Homogenates and subcellular fractions were stored at −10° until required for chemical and enzymatic analyses.

Enzymatic and Chemical Determinations

Assays for 5′-nucleotidase were carried out on the day following the preparation and for glucose-6-phosphatase and acid phosphatase, within four days of the date of preparation. These storage conditions were found to have no noticeable effect on the enzyme activities of the fractions.

Glucose-6-phosphatase was assayed according to the method of Hubscher and West.[21] The assay mixture contained 43 mM maleate buffer, pH 6·5, 4 mM EDTA, pH 6·5, 4 mM KF, pH 6·5, 28·5 mM glucose-6-phosphate and an aliquot of enzyme fraction in a total volume of 0·7 ml. The aliquots of tissue fractions used for all the enzyme assays varied from 0·1 to 0·3 ml depending upon their respective activities. Acid phosphatase was also measured as described by Hubscher and West;[21] the reaction mixture consisted of 50 mM acetate buffer, pH 5·4, 15 mM β-glycerophosphate, pH 5·4 and enzyme in a total volume of 2 ml. 5′-nucleotidase was assayed as described by Michell and Hawthorne[25] in a mixture containing 100 mM KCl, 10 mM $MgCl_2$, 50 mM Tris/HCl, pH 7·4, 10 mM NaK tartrate, 5 mM AMP and enzyme in a total volume of 2 ml. In a few instances, 4 mM EDTA and 4 mM KF were also included in the 5′-nucleotidase reaction mixture. For each enzyme, the reaction mixtures were incubated for 15 min at 37° and subsequently stopped with trichloroacetic acid (1 ml of 10%, w/v for glucose-6-phosphatase and 0·5 ml of 25%, w/v for acid phosphatase and 5′-nucleotidase) precooled to 4°. The precipitated protein was sedimented by centrifugation and aliquots of the resulting supernatants were analyzed quantitatively for inorganic phosphate according to the method of King.[26]

pH profiles of microsomal and soluble 5′-nucleotidase were determined by altering the pH of the assay system to that required except that the 50 mM Tris/HCl was replaced by 50 mM Tris/maleate for the pH range 5·0–7·0.

Protein determinations were routinely carried out by the method of Lowry *et al.*[27]

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