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Biochemical Properties of Potato Phosphorylase Change with its Intracellular Localization as Revealed by Immunological Methods*

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Abstract. Phosphorylase was purified from young and senescent potato tubers. Antibodies raised against the enzyme from young tubers crossreacted with phosphorylase from old tissue, although the latter exhibited different physico-chemical properties. In polyacrylamide gel electrophoresis it migrated with higher mobility, its subunit molecular weight was determined in the range of 40,000 in contrast to 100,000 of the phosphorylase in young tubers. The enzyme of senescent tubers displayed an isoelectric point of 5.4 different from the one of young tubers with 5.0, and the diffusion coefficients of the two enzymes varied. The appearance of the phosphorylase form typical for senescent tissue is connected with changes in the intracellular localization as revealed by immunofluorescence. Before massive starch accumulation is initiated, non-vacuolated subepidermal cells contain antigenically active material in their cytoplasm. During starch accumulation in fully differentiated storage parenchyma, only amyloplasts fluoresce, indicating the presence of adsorbed phosphorylase protein. Cytoplasmic phosphorylase can be detected in the continuance of senescence and, finally, after 16 months of tuber storage, the particle-bound enzyme had mostly disappeared. Simultaneously, we observed membrane destruction and decomposition on the ultrastructural level. The phosphorylase from senescent potatoes is a converted molecule and seems to be formed by proteolytic cleavage. The location of phosphorylase in the amyloplasts during starch synthesis indicates that it also plays a role in starch synthesis and not only in its degradation.

Key words: Amyloplasts – Enzyme conversion – Phosphorylose (starch) – Senescence – *Solanum* – Starch phosphorylase.

Abbreviations: PBS = phosphate buffered saline, FITC = fluorescein-isothiocyanate, IgG = immunoglobuline G

* Dedicated to Professor Dr. A. Frey-Wyssling on the occasion of his 80th birthday

Introduction

In plant storage tissues starch is synthesized in a special compartment, the amyloplast; this is different from animal tissues, where the corresponding storage polysaccharide, glycogen, is deposited in the cytoplasm in granular form. The molecular structure of the two polysaccharides is very similar. The same applies to the main enzymes involved in carbohydrate metabolism, phosphorylase and glycogen- or starch synthase (Meisel 1974; Fukui 1976). In animals it is clearly established that glycogen synthase catalyzes the formation of α-1,4-glycosidic linkages while phosphorylase is responsible for their cleavage (Fischer et al. 1971). Until now the role of phosphorylase in plants has remained questionable. There are several claims for the enzyme being involved in the synthesis of amylose chains as well (de Fekete and Vieweg 1974; Hawker et al. 1979). Plants were reported to contain a variety of phosphorylase isoenzymes, changing in dependence of the physiological state of the tissue investigated (Gerbrandy and Verleur 1971). In pea and spinach leaves, special isoenzymes could be attributed to the chloroplasts and cytoplasm, respectively (Steup and Latzko 1979). It was claimed that potato tubers also contain phosphorylase isoenzymes (Gerbrandy and Doorgeest 1972), though nothing is known about their intracellular distribution or physiological role. Changing isoenzyme patterns during development were partially explained by proteolytic cleavage of phosphorylase (Shivaram et al. 1971; Shivaram 1976). The physiological significance of the transition is still unclear. We intended to correlate biochemical and histochemical properties of the phosphorylases by simultaneous investigations of different physiological stages of the tissue.

Materials and Methods

Plant Material. Experiments were performed with potato tubers of different developmental stages: *young* tubers: mature potatoes harvested at a size of 3–5 cm diameter, processed immediately

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after harvest; *aging* tubers: fully grown, mature tubers, stored for up to 6 months after harvest at 4° C in the dark; *senescent* tubers: mature tubers, stored for more than 16 months at 4° C in the dark. Sieglinde and Hansa varieties were used in the experiments which showed no detectable differences.

Purification of Phosphorylase. All steps were performed at 4° C with buffers containing $6 \cdot 10^{-5}$ M benzamidine, $2 \cdot 10^{-4}$ M phenylmethylsulfonyl fluoride (PMSF), 10^{-5} M o-phenanthroline, $4 \cdot 10^{-3}$ M EDTA. Early harvests of *Solanum tuberosum* L. var. Sieglinde tubers were obtained from the local markets. Tuber homogenates were prepared from 2 kg of potatoes, homogenized with equal amounts (w/v) of 0.1 M Tris-HCl buffer, pH 8.4. After filtration ammonium sulfate was added to 37% saturation; after centrifugation the supernatant contained the phosphorylase, which was precipitated at 58% ammonium sulfate saturation. The sediment was dialyzed against 0.025 M Tris-HCl, pH 7.2, and chromatographed on a DEAE Cellulose (Whatman, U.K.) column (30·6.5 cm), previously equilibrated with Tris-HCl buffer. Phosphorylase was eluted by a linear NaCl gradient (100 to 400 mM NaCl). Active fractions were pooled, dialyzed against 0.025 M Tris-HCl with 125 mM NaCl and then applied to a DEAE Sephadex A-50 (Pharmacia, Sweden) column (40·2.5 cm). Enzyme activity was eluted by a linear NaCl gradient (175 to 300 mM NaCl) and concentrated by ultrafiltration through a PM 30 filter (Amicon, Corp., USA). After dialysis against 0.025 M Tris-HCl (pH 7.2), the concentrate was gel-filtered through a Sephadex G 200 (Pharmacia, Sweden) column (1.8·85 cm), as the final step of purification.

Polyacrylamide Gel Electrophoresis (PAGE). Native proteins were tested by electrophoresis in a Tris-aspartate PAGE system (Davis et al. 1967). Sodium dodecyl sulfate gels (SDS) were run as described by Weber and Osborn (1969), and disk gels were done according to Maurer (1968) using a Tris-glycine system and 5% acrylamide. Isoelectric focusing was carried out with the LKB plate system (Haglund 1971) at a maximal voltage of 1,500 V. The pH gradient was prepared by a mixture of three ampholine stem solutions (LKB, Sweden) (pH 3.5–5.0; 5.0–7.0; 7.0–10.0). Isoelectric points of different proteins were determined by marker proteins as proposed by Bours (1973). Phosphorylase activity in the gels was identified by incubation of the gels in 0.05 M glucose-1-phosphate (G-1-P) reaction mixture containing 0.02% glycogen, followed by KI_3 staining of synthesized amylose chains. Antigenic material separated in PAGE was detected after incubating the gels in antiserum dilution optimized for immune complex precipitation.

For this purpose gels had to be washed previously in phosphate buffered saline (PBS) 0.9% NaCl, 0.05 M sodium phosphate, pH 7.4). Precipitates could be detected after 48 h incubation at 4° C.

Protein Determination. The Biuret method (Gornall et al. 1949) was applied to extracts with a protein content ranging between 5 and 30 mg ml^{-1} with bovine serum albumine (Sigma Biochem., FRG) as a standard. At lower concentrations the specific absorbance of the solution at 260 and 280 nm (Warburg and Christian 1941), or the Coomassie G 250 method (Sedmak and Grossberg 1977), were applied. Pure enzyme preparations were quantified spectrophotometrically by the specific extinction coefficient $E_{1\%}^{280} = 11{,}7$ (Lee 1966).

Isolation of Amyloplasts. Amyloplasts from potato parenchyma were isolated from osmotically shocked and cleaved protoplasts prepared by the method of Wagner and Siegelman (1975) in 2% Cellulysine (Calbiochem, USA), 0.4 M mannitol in 5 mM sodium phosphate buffer, pH 5.8. Nylon mesh filtration (pore size: 55 µm) resulted in a suspension rather free from fibrillar material. Amyloplasts were then washed several times after free sedimentation in a cylinder and were microscopically free from other cellular components. The organelle diameter ranged from 15 to 60 µm.

Enzyme Activity Measurements. Phosphorylase activity was determined by measuring the liberation of phosphate from glucose-1-phosphate (Hedrick and Fischer 1965) with the following solutions: 0.05 M glucose-1-phosphate (Sigma Biochem., FRG) in 0.15 M sodium citrate buffer, pH 6.0, and 0.02% glycogen. The reaction was started by addition of 0.1 ml enzyme solution to 0.1 ml of the mixture. After incubation for 3 and 5 min at 30° C, the reaction was stopped by 50 µl of sodium dodecyl sulfate (10%) and liberated phosphate was assayed.

Preparation and Testing of Antibodies. Highly purified phosphorylase (3 mg), free from contaminants detectable by gel electrophoresis, were injected i.m. into 7-month-old outbred rabbits with complete Freund's adjuvans. The animals were boosted several times at 10- to 14-day intervals (about 2 mg protein each) in incomplete adjuvans. Antibody titers were sufficient after 8 to 12 weeks of immunization. The antiserum of highest specificity was selected and then separated by DEAE cellulose chromatography (Nabielek 1966). The IgG fraction contained the majority of antibodies directed to phosphorylase. It was further used for the histochemical tests and L-plate diffusion. The sera and γ-globulines were tested for monospecificity and titer by double diffusion in Ouchterlony plates (1% agar, Behring, FRG, in sodium barbital buffer, pH 7.4). The plates were incubated for 20 h at 4° C (Ouchterlony 1967).

Preparation of Plant Tissues for Immunohistochemistry. Light microscopy: Tissue slices (2 mm thick or less) were taken from several regions of the tuber and immediately immersed in fixation solution (3.7% formaldehyde, freshly prepared from paraformaldehyde, in 0.1 M sodium phosphate buffer, pH 7.0) for 2 h. After extensive washing in buffer, dehydration was performed by the continuous addition of 2,2-dimethoxypropane (Lin et al. 1977). The slices were finally embedded in paraffine about 10 to 15 h after the last dehydration step. Tissue sections were slightly stretched on gelatinized slides. After rehydration, the slides were immersed in phosphate buffered saline, pH 7.4, for 10 min, if later used in the immunohistochemical test.

Electron microscopy: A fixation procedure as proposed by Mohr and Cocking (1968) turned out to be most suitable for highly vacuolated plant tissue. Fixation was achieved in 2% glutardialdehyde in 0.02 M sodium phosphate buffer, pH 7.0. All steps including dehydration in alcohol were performed in an ice bath. Tissue pieces were finally embedded in styrene methacrylate and polymerized (12 h at 40° C, 24 h at 50° C and 24 h at 60° C). For preparation of ultrathin sections we used a Reichert OmU 2 microtome. The sections were poststained by uranyl acetate and lead citrate. Electron microscopy was performed in a Siemens Elmiskop IA.

Immunohistochemical Test. Paraffine sections (3–5 µm thick) were incubated in anti-phosphorylase or pre-immune IgG solution (20 to 50 µg protein ml^{-1}) for 2 h at 4° C. Free protein was removed by washing the slides in phosphate buffered saline (PBS), pH 7.4, for 30 min in a continuous buffer flow. Fluorescein-isothiocyanate (FITC) conjugated anti-rabbit IgG raised in goats (Flow Lab., FRG) diluted 1:20 was applied and again incubated for 2 h at 4°. Non-specific fluorescence was removed by extensive washing. The sections were examined in PBS buffered glycerol (1:9). Amyloplasts suspensions were treated similarly except that antibody to phosphorylase and anti-rabbit FITC were applied in suboptimal quantities in order to reduce washing to a minimum and avoid damage of the plastidal envelope. Binding to phosphorylase was identified by fluorescence and determined by microscopy using

a SM Lux Leitz epiflurorescence microscope. Photographs were taken on Kodak Ektachrome High speed 23 or Ilford IP 5.

L-Plate Diffusion. An agar layer about 3 mm deep on flat glass plates (5·5 cm) was prepared with two rectangular troughs at right angles. Equal volumes of antigen and antibody (IgG solution) were placed in the troughs containing equivalent amounts of reacting molecules (Allison and Humphrey 1960). Diffusion occured at 4° C. The angle of the straight precipitation line with the antigen-containing trough is related to the diffusion constants ratio of the participating molecules.

Results

Microscopical Studies. Our primary approach for the evaluation of phosphorylase reactions was the intracellular localization of the enzyme in different developmental stages of the potato tuber parenchymas. In order to exclude histochemical artefacts, tests performed with fixed plant material were also done using unfixed organelle preparations.

Antigen Distribution During Development of the Tuber. Enlargement of potato stolon (up to 7 mm in diameter) is accomplished by procambial cell divisions: Starch storing parenchyma cells (cortex layers) occur between epidermis and vascular ring (Reeve et al. 1970). The cortex cells contain starch-filled amyloplasts. Subepidermal cells were still without amyloplasts, as detectable by light microscopy, though they contain proplastids as reported by Marinos (1967) from electron microscopical investigations.

In the immunohistochemical test we found anti-phosphorylase receptors in the cytoplasm of these cells. Binding to any subcellular structures could not be observed (Figs. 1a, b). Controls performed with pre-immune rabbit serum were negative (Figs. 2a, b) and rabbit IgG unspecifically adsorbed to the plant material could not be detected. Fully differentiated storage cells in young potatoes (3–6 cm in diameter) contain large starch-filled amyloplasts in the cortex and perimedullary (Reeve et al. 1970). Tissue sections from such tubers, fixed immediately after harvest, specifically bound the antibody at the plastidal surface, as indicated by fluorescence (Fig. 3). After prolonged incubation the starch grain itself exhibits fluorescence (Fig. 4). The cytoplasm of such cells did not contain antigenic material, but a small amount of antigen could be detected at the envelopes of the organelles and at the plasmalemma.

In sections near the vascular bundle we found a negative antigenic reaction in the relatively small amyloplasts of the starch sheath cells. They did not contain the phosphorylase to which our antiserum had been directed (Fig. 5).

Amyloplasts isolated from the parenchyma protoplasts were tested unfixed in order to define the location of the antibody binding receptor more precisely. Best antibody binding occurs at alkaline pH and relatively high molarity of the dilution buffer. These conditions, however, favor the dissociation of phosphorylase from the plastids or starch grains (de Fekete 1966) and injure the plastidal envelope. In spite of this we succeeded in detecting at least part of the enzyme still associated with the isolated organelles. We observed specific capping of the antigen by the fluorescein conjugated antibody complex (Fig. 6) after incubation at 4° C followed by 30° C. Capping

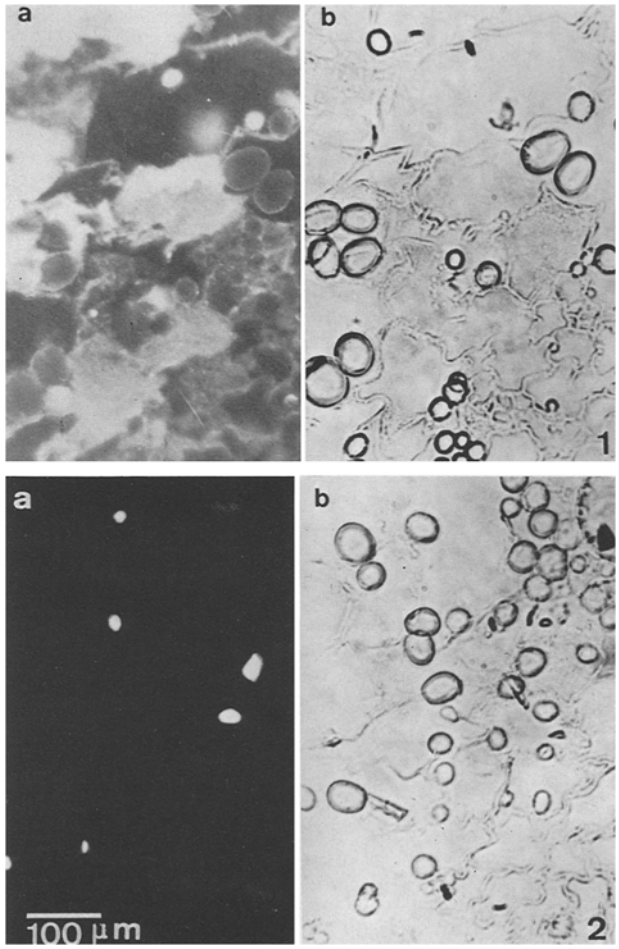


Fig. 1a and b. Tangential paraffine section (3 µm thick) of a very young potato (7 mm in diameter) showing subepidermal and cortex parenchyma cells after anti-phosphorylase (diluted 1:20 in PBS) treatment followed by anti-rabbit IgG-FITC decoration (1:10 in PBS). Fluorescence is located in the cytoplasm of the subepidermal cells and at the amyloplasts' surface of the cortex cells. **a** Fluorescence micrograph, **b** corresponding light micrograph

Fig. 2a and b. Tangential paraffine section (3 µm thick) as in Fig. 1, but treated with pre-immune rabbit serum (diluted 1:10 in PBS) followed by anti-rabbit IgG-FITC (1:10 in PBS). This control experiment shows that no serum IgG is unspecifically adsorbed by the plant material. **a** Fluorescence micrograph, **b** corresponding light micrograph

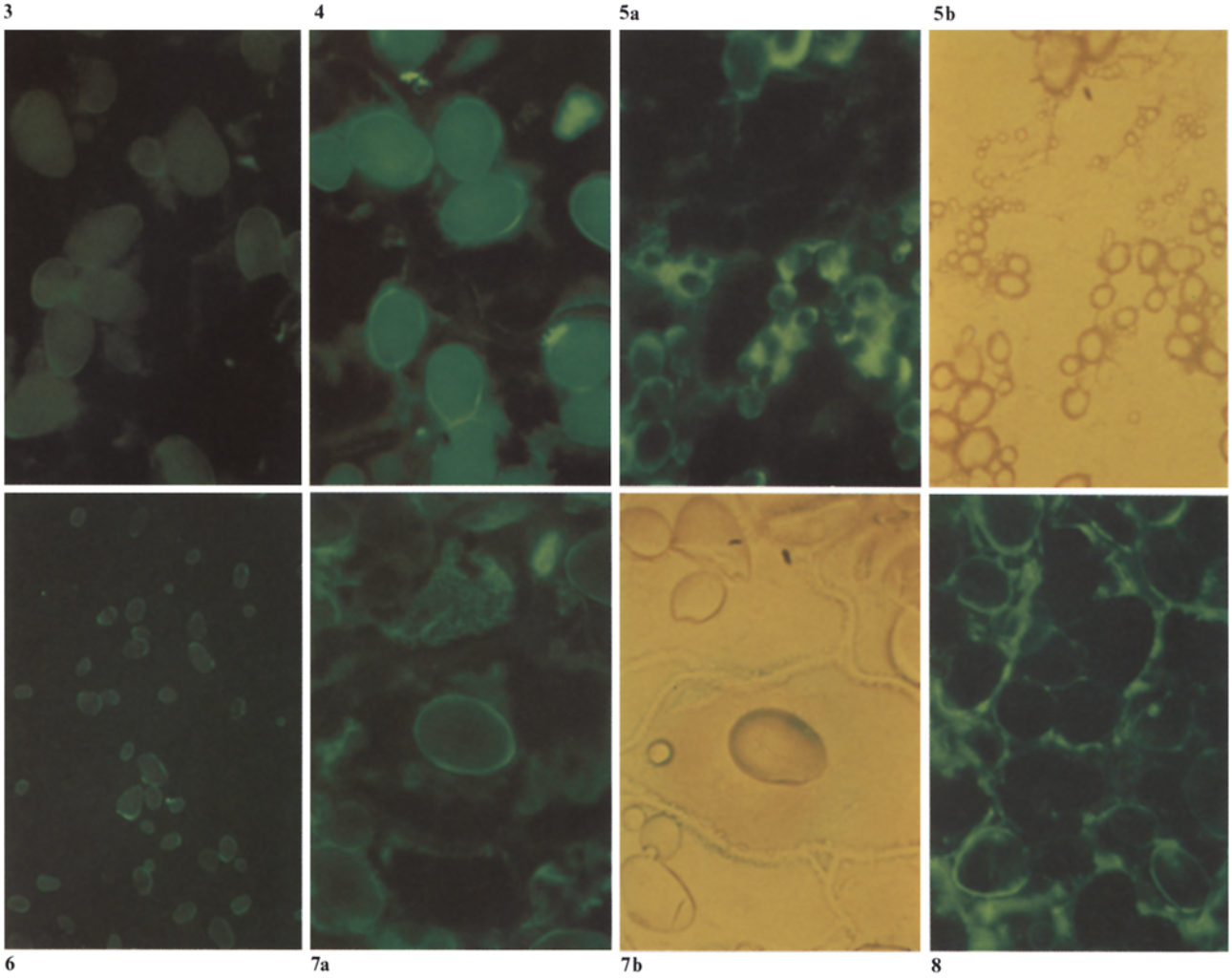


Fig. 3. Fluorescence micrograph of differentiated potato storage tissue, fixed immediately after harvest. The section (5 μm) had been treated with anti-phosphorylase dilution and anti-rabbit IgG as indicated in Fig. 1. The surface of the amyloplasts is primarily decorated by the antibody conjugate; in addition, there is positive fluorescence at the plasmalemma and the envelope of the plastids. (× 240)

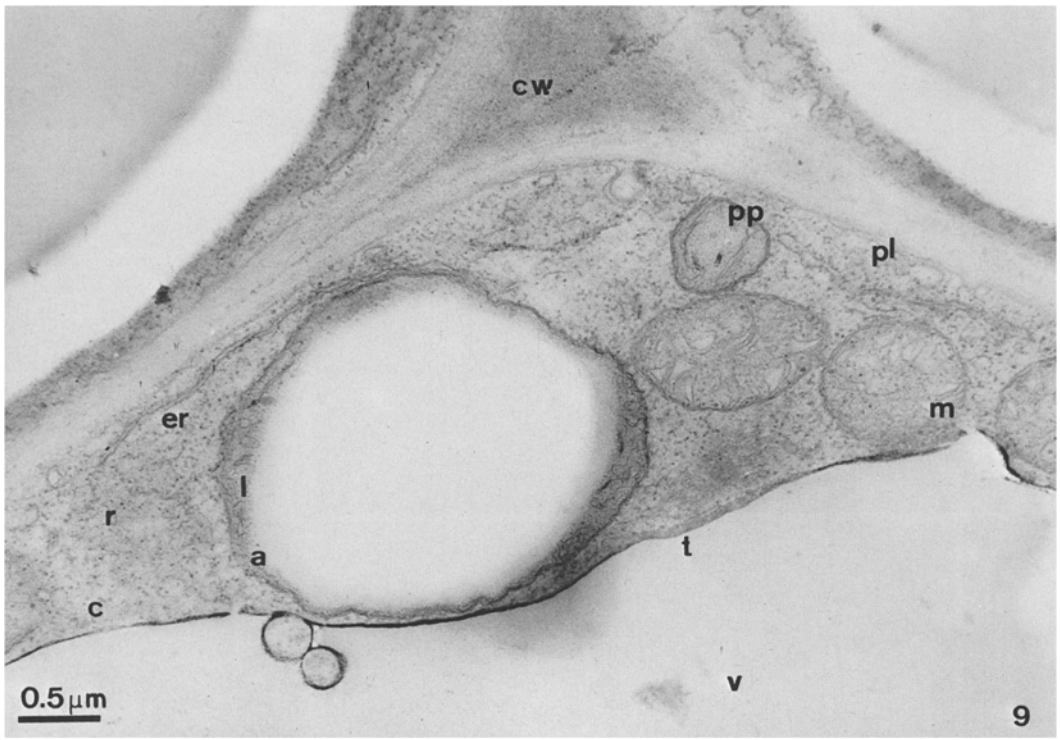
Fig. 4. Fluorescence micrograph of an identical sample as in Fig. 3 but the immunohistochemical test of the section differed in terms of the incubation time in anti-phosphorylase and the anti-rabbit IgG-FITC, which were applied for 6 h in contrast to 2 h in normally performed tests. The starch grains within the organelles are highly fluoresceing, indicating phosphorylase as a component of the starch molecule, though there is only limited access for the antibody to its antigen. (× 240)

Fig. 5a and b. Fluorescence micrograph (**a**) and transmitted light micrograph (**b**) of a potato tuber section near the vascular bundle. Amyloplasts in the starch storage parenchyma (large ones) are positive, whereas amyloplasts of the bundle sheath cells (small ones) show no fluorescence. (× 240)

Fig. 6. Unfixed amyloplast preparation of potato tubers. The incubation with anti-phosphorylase serum was performed at 4° C for 2 h followed by 30° C incubation for 4 h. Capped receptor molecules at the plastidal envelope were decorated by the FITC complex. The starch grains remained dark as the membranes acted as diffusion barriers for IgG. (× 60)

Fig. 7a and b. Localization of phosphorylase in potato storage tissue which had been stored for six months before fixation. Comparing the fluorescence micrograph with the light micrograph (**a** and **b**) not all amyloplasts in the section have phosphorylase, but there is a considerable amount of antigen in the cytoplasm. (× 240)

Fig. 8. Localization of phosphorylase in potato storage parenchyma after 16 months of storage. The solubilization of phosphorylase antigen is more prominent than after 6 months of storage (cf. Fig. 7). Most amyloplasts are surrounded by a fluoresceing matrix and positive stain at the envelope seems doubtful. (× 240)



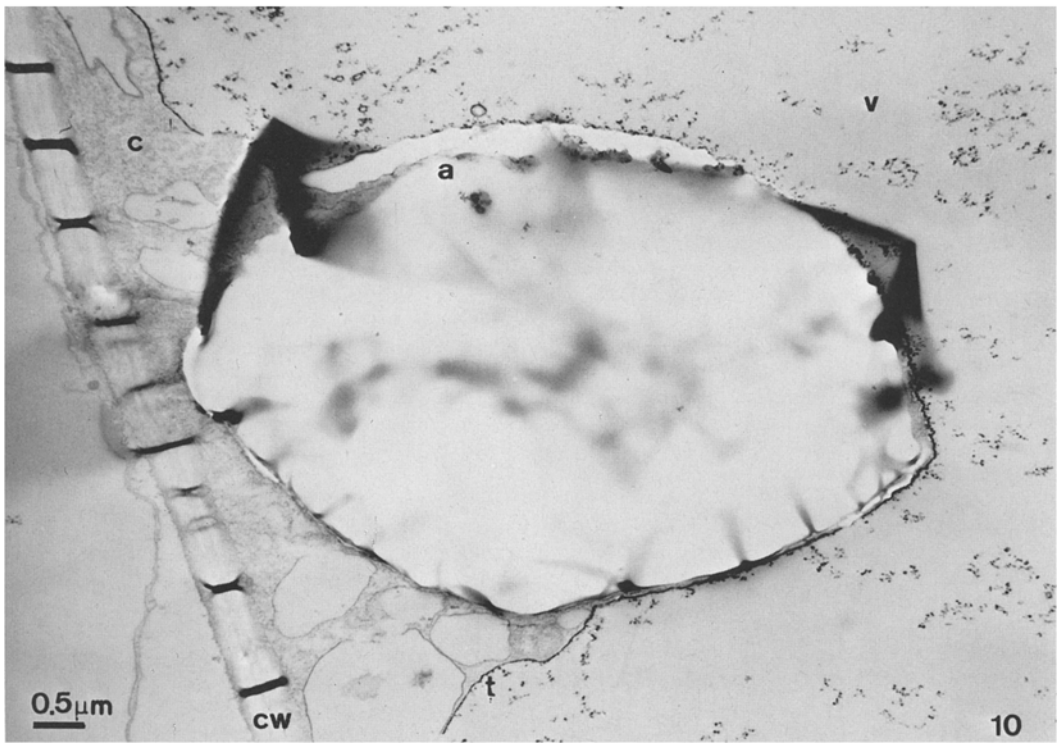


Fig. 9. Electron micrograph of differentiated storage parenchyma (variety: Sieglinde), fixed immediately after harvest in 2% glutardialdehyde, 0.05 M sodium phosphate; the section was poststained by uranyl acetate and lead citrate. The cells are highly vacuolated and amyloplasts (*a*) are almost filled with starch, their inner envelope membrane has formed tubular invaginations (*l*). The plastids are often found in tight association to the endoplasmic reticulum (*er*). The cytoplasm (*c*) contains mitochondria (*m*), proplastids (*pp*), and ribosomes (*r*). Tonoplast (*t*), cell wall (*cw*), vacuole (*v*), plasmalemma (*pl*)

Fig. 10. Electron micrograph of aged tuber parenchyma (variety: Sieglinde) fixed after 16 months of storage at 4° C in the dark, preparation was as indicated for young tubers (cf. Fig. 9). It is crucial to distinguish the plasmalemma from other membranes, ie. tonoplast and residual envelopes. Membrane vesiculation further stresses decompartimentation of the cells. (Abbreviations as indicated for Fig. 9)

indicates that the antigen is membrane-associated (de Petris and Raff 1973).

Antigen Distribution During Potato Tuber Senescence. After 6 months of tuber storage at 4° C the binding of the antibody to amyloplasts in fixed sections was less prominent (Fig. 7), in comparison to young tuber sections (Figs. 3, 4). Some amyloplasts were completely negative (cf. Figs. 7a and b); simultaneously, antigen appeared in the cytoplasm of the cells. Prolonged storage of the tubers further diminished the amount of positive plastids in the tissue. After 16 months of storage, there are only very few organelles showing fluorescence at their surface; most antigenically active material must be attributed to a cytoplasmic matrix (Fig. 8) actually surrounding the amyloplasts or starch grains. This suggested membrane degradation in the aged potato parenchyma. Therefore, we studied the different physiological stages of potato by electron microscopy.

Ultrastructure of Young and Senescent Storage Parenchyma. Several studies have led to divergent interpretations of the ultrastructure in young and old storage cells of the potato (Ohad et al. 1971; Wetzstein and Sterling 1978), although results may depend on the tuber variety.

Ultrastructure of the starch storage cells in young and senescent tubers is presented in Figs. 9 and 10. Both tissues are highly vacuolated and their amyloplasts are completely filled with starch. In the case of young potato parenchyma (Fig. 9) which had been fixed immediately after harvest, a stroma space with tubular invaginations of the inner envelope membrane is visible in the amyloplasts. The cytoplasm is extremely rich in ribosomes and mitochondria; cisternae of the endoplasmic reticulum often appear in tight association to the plastids. The tissue is actively involved in starch biosynthesis as proplastids are also still present.

By contrast, parenchyma cells of senescent tubers stored for 16 months show beginning decomposition: The plasmalemma is highly vesiculated and the plastidal envelope does not continuously surround the starch grain (Fig. 10); it was often difficult to distinguish envelope from tonoplast. As we tested several fixation procedures and included variations in the molarity of the buffer and pH, we imply that decomposition is a process actually occurring in vivo.

Biochemical Studies. *Purification of Phosphorylase From Young and Senescent Potatoes.* Preparation of phosphorylase from young and senescent tubers was performed as described in materials and methods. The senescent tubers had not yet started sprouting. In comparison to the young tissue, the purification of aged tissue had to proceed very rapidly and with higher concentrations of mercaptoethanol (10^{-2} M) in the buffers, as highly active polyphenol oxidases yield inhibitory products for phosphorylase (Blank and Sondheimer 1969).

Elution patterns of the different chromatographic steps are shown in Figs. 11a–f. The activity was eluted by a linear NaCl gradient in the case of DEAE Cellulose and DEAE Sephadex A-50 chromatography. During the preparation of phosphorylase from young tissue, two peaks of activity were clearly separated on DEAE Cellulose and DEAE Sephadex A-50 chromatography, while only one peak appeared during purification of the enzyme from stored tubers. Gel filtration by Sephadex G 200 characterized the young tuber enzyme with 200,000 molecular weight (cf. Iwata and Fukui 1973). The purified phosphorylase of stored potatoes eluted later, at 160,000 molecular weight (Figs. 11c, f). Final preparations had a specific activity of about 12 U mg^{-1} protein.

Change of the Molecular Properties of Phosphorylase During Aging. We evaluated the changes concerning the molecular properties of phosphorylase purified from young and senescent tubers by a combination of different immunological and electrophoretical methods.

The Ouchterlony double diffusion agar plate (Fig. 12) clearly shows that the antiserum reacts only with one protein in homogenates of different potato varieties. In addition, there is a continuous precipitation line between the wells of young and senescent tuber samples.

Figure 13 shows the phosphorylase activity after isoelectric focusing of the DEAE Cellulose eluates of homogenates from young tubers (Fig. 13a), aging tubers (Fig. 13b), and from senescent tubers (Fig. 13c). The preparation of young tubers exhibited one major activity band at pH 5.0; the sample of six-month-old tubers gave rise to two activity bands: one at pH 5.0 and a second one at 5.3 to 5.4; senescent tubers contained only the activity which focused at pH 5.3 to 5.4.

Since ampholine gels are not suitable for immune complex precipitation, we identified the different phosphorylases by means of antibody precipitation in disk gels (Maurer 1968). This system made it possible to distinguish two closely spaced bands in the case of phosphorylase from aging potatoes by means of immune complex precipitation (Fig. 14 gel: 1). The sample prepared from young tubers, however, yielded only one precipitate (Fig. 14 gel: 3) under the same conditions. The two protein moieties could not be resolved by activity staining (Fig. 14 gels: 2 and 4),

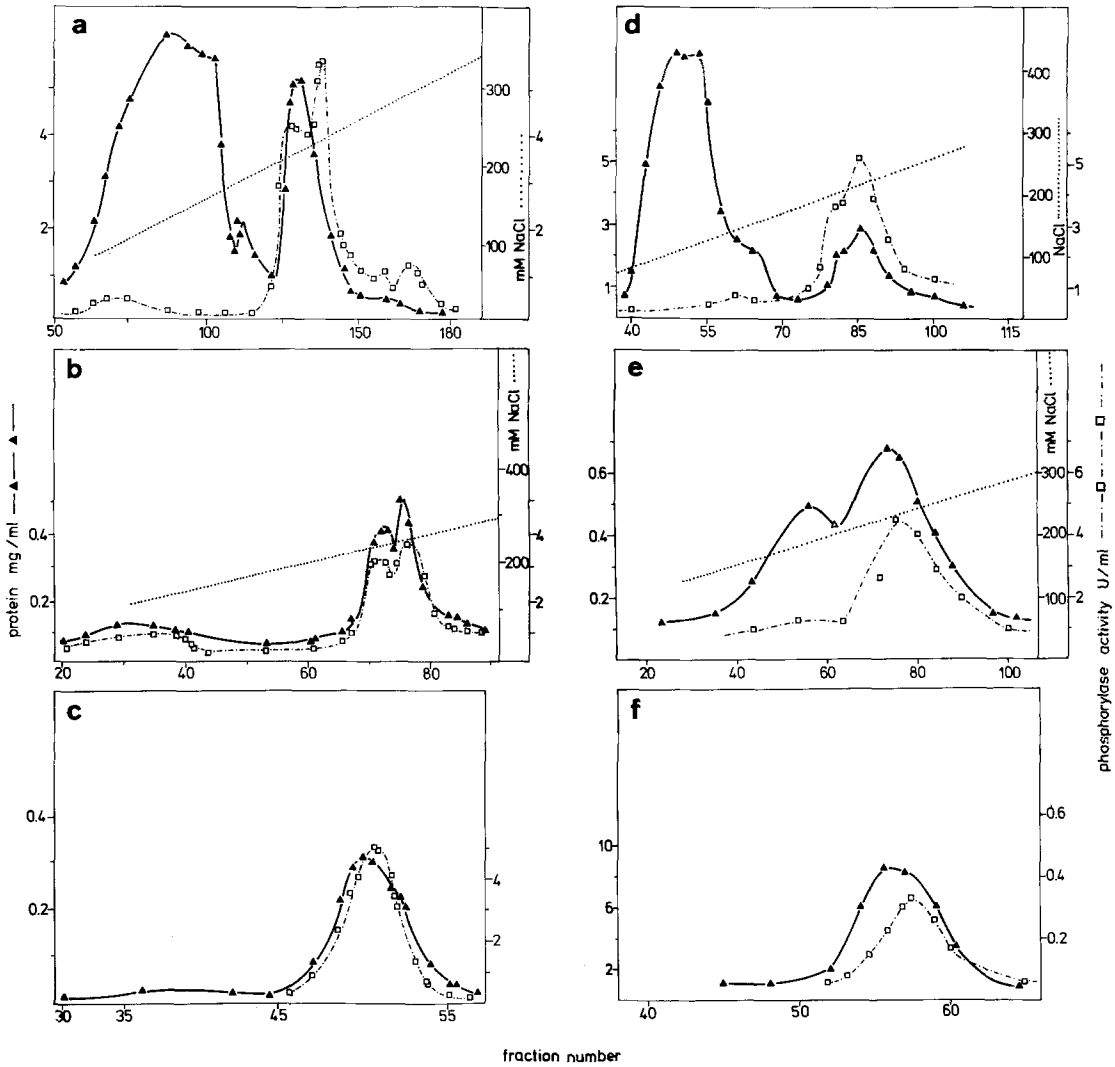


Fig. 11a–f. Elution patterns of the different chromatographic steps of phosphorylase purification from potato (variety: Sieglinde). Tubers extracted immediately after harvest (**a–c**); tubers extracted after 16 months of storage (**d–f**). **a** DEAE Cellulose chromatogram: the linear NaCl gradient elutes the main activity in a double peak between 205 and 240 mM. The active fractions were pooled for further steps (15 ml/fraction) **b** DEAE Sephadex A-50 chromatogram: the double activity peak correlated with a double protein peak. Fractions between 215 and 235 mM NaCl of the linear gradient were collected (5 ml/fraction) **c** Sephadex G 200 gel chromatogram: 4 mg protein of the DEAE Sephadex A-50 eluate were separated per run (0.8 ml/fraction) **d** DEAE Cellulose chromatogram: the main activity eluted here as a single peak and correlated with the major protein peak. Fractions between 200 and 220 mM NaCl of the linear gradient were pooled (18 ml/fraction) **e** DEAE Sephadex A-50 chromatogram: the main activity and protein eluted between 220 and 260 mM NaCl (5 ml/fraction) **f** Sephadex G-200 gel chromatogram: 4 mg protein of the DEAE Sephadex A-50 active eluate were separated per run. Phosphorylase eluted later than the enzyme from young tubers (cf. 11 c) (0.8 ml/fraction)

only a widening of the activity band is observed in extracts from aged potatoes. For further characterization of the two phosphorylase activities, the enzyme was purified from young and senescent potatoes (Figs. 11a–f). Results are presented in Figs. 15a and b. By gel electrophoresis in the presence of sodium dodecylsulfate, the subunit molecular weight of phosphorylase from young tubers was determined as 100,000 molecular weight while the enzyme in aged tubers was found in a wide band around 40,000 molecular weight (Fig. 15a). The biochemical transition between the two forms of phosphorylase activity is reflected in the immunohistochemical results (Fig. 7). where antigenically active protein was found in the amyloplasts and the cytoplasm as well. After 16 months of storage, the high molecular weight enzyme present in young tubers had disappeared (cf. Fig. 13c), coinciding with cytoplasmic staining in the immunofluorescence assay (Fig. 8).

Diffusion Coefficients Determined in L-Plates. The two phosphorylase forms were also different concerning

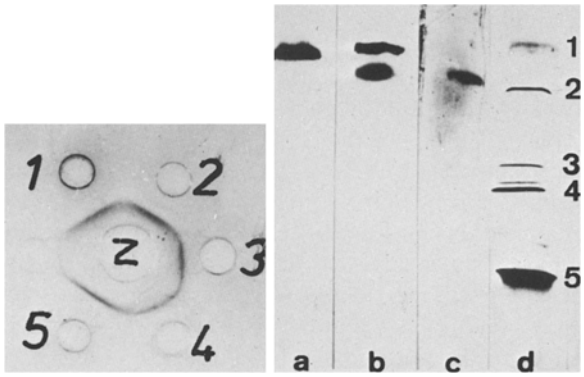


Fig. 12. Ouchterlony double diffusion assay performed with homogenates of different potato varieties. The central well (*Z*) contained the phosphorylase antiserum (diluted 1:5 in PBS) and the surrounding wells tuber homogenates of the varieties Hansa (*1*), Saskia (*2*), Sieglinde (*3*), Hansa (*4*) and Sirtema (*5*). Samples 1, 2, 3, and 5 were obtained from young tubers; sample 4 from tubers stored for 16 months. The wells contained 20 µl each; diffusion occured at 4° C, and precipitation lines developed after 20 h of incubation

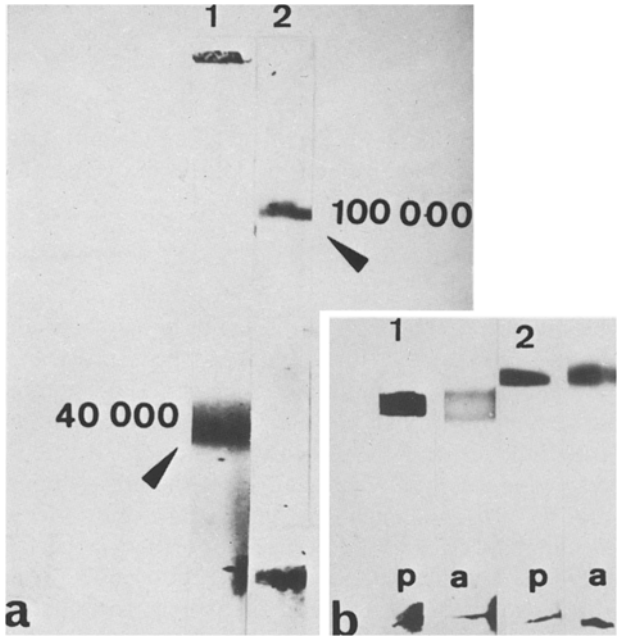


Fig. 15a and b. Gel electrophoresis of purified phosphorylase preparations (0.05 mg protein/run). **a** Electrophoresis in the presence of sodium dodecyl sulfate (SDS); gel *1*: phosphorylase obtained from aged tubers: the molecular weight of the subunits was determined in the range of 40,000; gel *2*: corresponding phosphorylase from young tubers; the molecular weight of the subunits was 100,000. **b** Gel electrophoresis of native phosphorylase by the Tris-aspartate system; gel *1*: aged tuber phosphorylase; gel *2*: young tuber phosphorylase. The electrophoretic mobility of the aged tuber enzyme is higher compared to the one prepared from young potatoes. (*p*=protein stain by Coomassie G 250; *a*=activity stain by KI_3 after G-1-P incubation, cf. Fig. 13)

Fig. 13. Isoelectric focusing of the pooled phosphorylase active fractions after DEAE Cellulose chromatography. The gels were incubated in a glucose-1-phosphate solution (0.05 M glucose-1-phosphate, 0.02% glycogen in 0.15 M sodium citrate buffer, pH 6.0); synthesized amylose chains were stained by KI_3 (about 0.4 mg protein/run; variety Sieglinde). *a*=young potato tuber eluate; *b*=tuber eluate of six months stored potatoes; *c*=tuber eluate of aged, i.e. 16 months stored potatoes; *d*=marker proteins (stained with Coomassie G 250). *1*=albumine (IEP 4.95); *2*=β-lactoglobuline A (IEP 5.4); *3*=carbonic acid anhydrase (IEP 6.18); *4*=myoglobine, small subunit (IEP 7.3); *5*=myoglobine, large subunit (IEP 7.58) (Bours 1973)

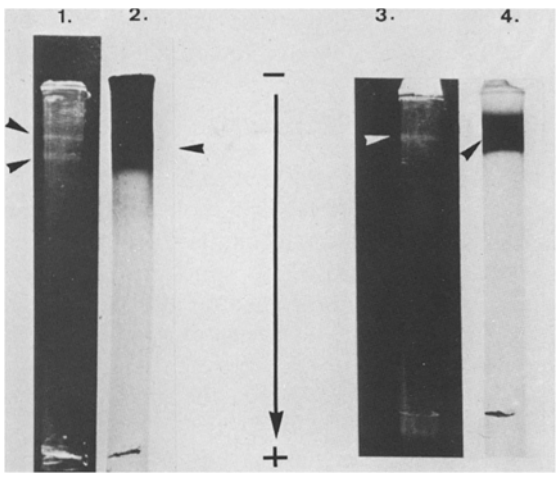


Fig. 14. Immune precipitation of DEAE Cellulose eluates after separation by disk gel electrophoresis (5% polyacrylamide, Tris-glycine system, 0.1 mg protein per run); gels *1, 3* were incubated in antiserum (diluted 1:20 in PBS for 48 h); gels *2, 4* in G-1-P solution for 6 h (cf. Fig. 13). Two antigenically active protein moieties could be distinguished by immune complex precipitation in the case of six months stored tuber samples (gel *1*) in contrast to one band in the sample obtained from young tubers (gel *3*). The activity stain (gels *2* and *4*) was not informative in this system

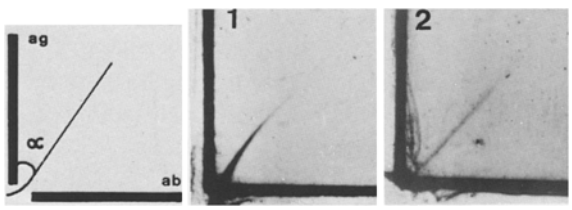


Fig. 16. Determination of diffusion coefficients by L-plate diffusion according to the schematic drawing. The precipitate of the young tuber phosphorylase with anti-phosphorylase IgG occured at an angle of 36° (plate *1*), in contrast to the one of aged tuber phosphorylase at an angle of 43° (plate *2*) (0.05 mg phosphorylase/trough; 0.2 mg anti-phosphorylase IgG/trough)

their diffusion properties as tested by the L-plate diffusion technique (Fig. 16; Allison and Humphrey 1960). The diffusion constant for converted phosphorylase was higher ($3.3 \cdot 10^{-7}$ cm^2 s^{-1}), according to an angle of 43° between the antigen-containing trough and the precipitation line than the constant calculated for phosphorylase from young tubers ($2.0 \cdot 10^{-7}$ cm^2 s^{-1}) with an angle of 36°. The diffusion constant of an antigenically active protein is related to the angle according to the following equation:

$$\tan \alpha = \sqrt{\frac{D_{ag}}{D_{ab}}}.$$

The diffusion constant (D_{ab}) of IgG, i.e., the antibody population, was determined by Allison and Humphrey (1959) to be $3.8 \cdot 10^{-7}$ cm^2 s^{-1}.

Discussion

The characterization of multiple forms of α-1,4-glucan phosphorylases in various plants has been a general problem in the evaluation of phosphorylase action on carbohydrate metabolism. Our results suggest a proteolytic cleavage of possibly one major native enzyme with accompanying changes of the intracellular location of the enzyme during aging. The multiplicity of phosphorylases has been attributed to special stages of development as in maize endosperm (Tsai and Nelson 1968, 1969) and in banana fruit pulps (Singh and Sanwal 1975). In the photosynthetically active tissue of pea and spinach, Steup and Latzko (1979) succeeded in clearly defining cytoplasmic and plastidal phosphorylases.

The identity of phosphorylase isoenzymes in the storage tissue of the potato, however, has remained questionable. Shivaram et al. (1971) and Shivaram (1976) reported two isoenzymes and Gerbrandy detected many more in tubers (Gerbrandy 1974). Since migration of phosphorylase can be influenced by adsorbed primer molecules and posttranslational processing of the polypeptide chain (Iwata and Fukui 1973, 1975; Gerbrandy 1974), the appearance of multiple activity bands, alone, is no proof of the presence of isoenzymes. We characterized one phosphorylase protein by monospecific antibodies in young and senescent tubers of several potato varieties: The precipitation lines in the Ouchterlony double diffusion assay (Fig. 12) entirely fused, which suggests that in all samples only one identical antigen was recognized and precipitated (Ouchterlony 1967).

Under certain conditions, however, phosphorylase gives rise to two bands in polyacrylamide gel electrophoresis (Fig. 14). Investigations of the physico-chemical properties of both forms, purified from young and senescent tubers, showed that they corresponded to the enzyme described by Shivaram (1976) and its conversion product (cf. Figs. 13 and 15), generated by proteolytic cleavage of phosphorylase during senescence. Iwata and Fukui (1973) found similar phenomena in partially purified phosphorylase preparations.

Determination of the intracellular localization of phosphorylase by immunofluorescence pointed to a connection between phosphorylase conversion and transition of starch metabolism from synthesis to breakdown.

At the beginning of potato tuber differentiation, phosphorylase is homogeneously distributed in the cytoplasm (Fig. 1) of the cells (Schneider et al. 1979). Hence, phosphorylase or phosphorylase precursor molecules can be detected in the cytoplasm of parenchyma cells before the differentiation of proplastids to amyloplasts is achieved (cf. Marinos 1967). In differentiated storage tissue of the cortex and perimedullary, amyloplasts themselves were specifically decorated by the antibody-FITC conjugate (Figs. 3, 4, 5); the amyloplasts of the vascular bundle sheath cells, however, were negative in antibody-binding, proving that their phosphorylase was different from or lacking the enzyme in storage tissue (Fig. 5). Ultrastructural studies of mature potato storage parenchyma showed that the cells still contain proplastids besides larger amyloplasts. The organelles are often found in tight association to the endoplasmic reticulum (Fig. 9). This is considered as metabolic transport system between cytoplasmic and plastidal compartments (Rodrìguez-Garcìa and Sievers 1977). We, therefore, imply a strong metabolic exchange to be still present in mature parenchyma, though this conclusion has been achieved indirectly.

In contrast to very young cells (cf. Fig. 1), we found phosphorylase in mature cells inside the amyloplasts and partially at the plasmalemma. This confirms observations of Thomas et al. (1979) who studied the location of phosphorylase in maize bundle sheath cells by a histochemical method (lead precipitation). These authors also observed binding of phosphorylase to the organelles and membranes, including the plasmalemma.

Therefore, membrane affinity of phosphorylase seems to be a general property of this protein, and it should be discussed in relation to the physiological role of the enzyme in starch metabolism. The intraplastidal location in starch-accumulating potatoes favors the anabolic action of phosphorylase catalysis. In the case of maize bundle sheath chloroplasts, this action of phosphorylase has been well established (de Fekete 1966; de Fekete and Vieweg 1974), its role in potato storage parenchyma is still a matter of discussion (Hawker et al. 1979).

Chain elongation in the plastids possibly occurs from fixed points at the envelope where synthesizing enzymes are located (Hawker et al. 1979). As soon as a certain chain length is obtained, the enzymes are precipitated onto the grain and included at random in the starch (Smith et al. 1968). Thus the relatively high protein content of the starch grain and the identification of phosphorylase in the grain itself, detected by our method (Fig. 4), can be explained.

The affinity of phosphorylase for the amyloplast membranes is documented by the capping of the enzyme, induced by binding of the antibody (Fig. 6). The capping phenomenon occurs only if the antigen is situated in the fluid lipid system of a biological membrane (de Petris and Raff 1973).

With aging of the potato tubers the intracellular distribution of phosphorylase changes: It is increasingly found in the cytoplasm of the parenchyma cells (Figs. 7 and 8). Our ultrastructural studies prove that amyloplast membranes are damaged at that time and the phosphorylase may passively diffuse into the cytoplasm (Fig. 10). It seems reasonable to assume that vacuolar and cytoplasmic proteases now act on plastidal proteins such as phosphorylase and bring about phosphorylase conversion. A proteolytic cleavage of phosphorylase is involved, though we cannot decide whether this is a specific or unspecific reaction. The 'nicked' enzyme exhibits different chromatographic properties (Fig. 11) and its diffusion coefficient is increased in comparison to the one of the original enzyme.

Our ultrastructural studies have shown that the tonoplast is also involved in the decompartimentation process that occurs during senescence of the tuber. This would lead to an increase of inorganic phosphate released from the vacuole (Loughman 1960) or set free by acid phosphatase activity. Inorganic phosphate facilitates dispersion of starch (Erlander and Tobin 1967) and attack by catabolically active enzymes. The equilibrium constant of the phosphorylase reaction

$$(\alpha\text{-1,4-polyglucan})_n + P_i \rightleftharpoons \text{glucose-1-phosphate} + (\alpha\text{-1,4-polyglucan})_{n-1}$$

(Meisel 1974) is close to unity. Therefore, its direction strongly depends on the P_i/G-1-P ratio; thus, a high ratio would promote amylose chain degradation mediated by phosphorylase.

In addition, Schilling and Dittrich (1979) have shown that complete starch breakdown is only accomplished if phosphorylase accompanies the action of amylases; this can further explain the fact that isolated starch grains cannot be degraded in vitro by addition of amylases alone (Meisel 1974). We suggest that the phosphorylase conversion is related to aging and concomittant membrane degradation. The observed changes in the location of phosphorylase correlate well with the ultrastructural alteration and with a transition in the biochemical behavior of phosphorylase. The transition probably provides a switch between the anabolic metabolism of the growing tuber and the catabolic metabolism of the aging one.

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